FERNANDA PEREIRA DA SILVA

VIRULÊNCIA E RESISTÊNCIA DE *Salmonella enterica* EXPOSTA AO PEPTÍDEO ANTIMICROBIANO NISINA

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

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(Coorientadora)

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(Orientadora)

DEDICATÓRIA

A Deus;

Ao meu pai Manoel, (in memoriam);

À minha família;

E, com toda gratidão,

À minha mãe, Dijanira (Dja),

Pela grandeza de sua alma,

Seu trabalho árduo,

Amor incondicional,

Apoio e compreensão,

Dedico!

Não coloque limites em seus sonhos,
coloque Fé!

(Papa Francisco)

"Quem sabe faz a hora, não espera
acontecer! "

(Geraldo Vandré)

"Para ter sucesso, alguém, algum dia, teve
que tomar uma atitude de coragem! "

(Peter Drucker)

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BIOGRAFIA DA AUTORA

FERNANDA PEREIRA DA SILVA, filha de Dijanira Z. Pereira da Silva, Manoel Pereira da Silva (*in memoriam*) e Miguel de Souza Magalhães, nasceu em Tangará da Serra - MT, em 26 de novembro de 1986. Em agosto de 2006, ingressou no curso de Licenciatura Plena e Bacharelado em Ciências Biológicas na Universidade do Estado do Mato Grosso (UNEMAT), campus de Tangará da Serra-MT, graduando-se em junho de 2010 (Turma: *Tabebuia aurea*).

Em março de 2013 iniciou o mestrado no Programa de Pós-Graduação em Microbiologia Agrícola da Universidade Federal de Viçosa-UFV, defendendo a dissertação em fevereiro de 2015, com o projeto sobre "Identificação de vírus a partir de bactérias fitopatogênica".

E por fim, em março de 2015 iniciou o Doutorado em Microbiologia Agrícola-UFV, onde desenvolveu pesquisa realizada na presente tese, com defesa em junho de 2019.

SUMÁRIO

RESUMO

SILVA, Fernanda Pereira, D.Sc., **Virulência e resistência de *Salmonella enterica* exposta ao peptídeo antimicrobiano nisina**. Universidade Federal de Viçosa, junho de 2019. Orientadora: Maria Cristina Dantas Vanetti. Coorientadores: Maria Emilene Martino Campos Galvão e Uelinton Manoel Pinto.

Salmonella enterica é um importante patógeno de origem alimentar de impacto global, que causa gastroenterite e febres entéricas. Alternativas para o controle do crescimento microbiano utilizando peptídeos antimicrobianos têm sido demonstradas ao longo dos anos e a nisina se destaca como uma substância considerada segura (GRAS). Nisina é um peptídeo catiônico que se liga ao lipídio II da membrana interna dos procariontes e tem sido intensamente utilizado no controle de bactérias Gram-positivas. O objetivo deste trabalho foi avaliar a ação da nisina no crescimento, formação de biofilme, expressão gênica, virulência e resistência de *Salmonella* Enteritidis PT4 578. O efeito inibidor da concentração sub-inibitória (sub-MIC) de nisina de 11,72 μM foi potencializado na presença de pH baixo (4,0; 4,5 e 5,5) e de NaCl (1,5, 2,5 e 5,0%), mas não na presença de 500 e 1000 μM de H_2O_2. Quando *S. enterica* foi exposta a concentrações sub-MIC de nisina de 11,72 μM e 46,88 μM, o perfil de alguns genes transcritos aumentou a expressão, como os genes de resistência (*pag*C), virulência (*inv*A e *inv*F) e formação de biofilme (*fim*F). O aumento da virulência de *S. enterica* promovido por concentrações sub-MIC de nisina foi confirmado em larvas de *Galleria mellonella*, que apresentaram maior taxa de mortalidade em menor tempo pós-inoculação e interferência na resposta imune celular e humoral, com redução do número de hemócitos totais, formação de nodulação e pseudópodes (protrusões), biossíntese de melanina (melanização) e regulação de proteínas de defesa (cactus e peroxidases). Estes resultados demonstram que agentes estressores potencializam o efeito da nisina no controle do crescimento de *S. enterica* e que nisina aumenta a virulência desse patógeno em um modelo animal.

ABSTRACT

SILVA, Fernanda Pereira, D.Sc., Universidade Federal de Viçosa, June, 2019. **Virulence and resistance of *Salmonella enterica* exposed to the antimicrobial peptide nisin**. Adviser: Maria Cristina Dantas Vanetti. Co-advisers: Maria Emilene Martino Campos-Galvão and Uelinton Manoel Pinto.

Salmonella enterica is an important food-borne pathogen of global impact, which causes gastroenteritis and enteric fevers. Alternatives for the control of microbial growth using antimicrobial peptides have been demonstrated over the years and nisin stands out as a substance considered safe (GRAS). Nisin is a cationic peptide that binds to lipid II of the inner membrane of prokaryotes and has been intensively used in the control of Gram-positive bacteria. The objective of this work was to evaluate the action of nisin on the growth, biofilm formation, gene expression, virulence and resistance of *Salmonella* Enteritidis PT4 578. The subinhibitory concentration (sub-MIC) of nisin was defined as being 11.72 μM its effect inhibitor was potentiated in the presence of agents such as low pH (5.5, 4.5 and 4.0) and high NaCl concentration (1.5, 2.5 and 5.0 %), but not in the presence of 500 and 1000 μM H_2O_2. When *S. enterica* was exposed to concentrations of nisin sub-MIC of 11.72 μM and 46.88 μM the profile of the transcribed genes was altered, such as virulence (*inv*A and *invF*) resistance genes (*pagC*) and formation of biofilm (*fimF*). The increased virulence of *S. enterica* promoted by sub-MIC concentrations of nisin was confirmed in *Galleria mellonella* larvae, which present a higher mortality rate in a short time after inoculation and evidence in the cellular and humoral immune response, with a reduction in the number of total hemocytes, nodulation formation, pseudopodia (protrusions), biosynthesis of melanin (melanization) and regulation of defense proteins (cactus and peroxidases). The results demonstrating that stressors potentiate the effect of nisin on the growth of *S. enterica* cells and increase the virulence of this pathogen upon an animal model.

INTRODUÇÃO GERAL

Salmonella spp. é uma bactéria Gram-negativa, móvel, pertence à família *Enterobacteriaceae*, conhecida mundialmente pela capacidade em causar gastroenterites e febres entéricas. Além de representar uma das principais zoonoses de importância para saúde pública em países subdesenvolvidos e desenvolvidos, é responsável por grandes perdas econômicas na indústria alimentícia, especialmente no setor aviário.

A salmonelose é uma doença entérica causada por sorovares de *Salmonella enterica* subsp. *enterica* não tifoides. O ciclo da doença em seres humanos inicia com a ingestão de alimentos ou água contaminados. A bactéria sobrevive à ação do suco gástrico estomacal e atinge o intestino, onde invade as células epiteliais e os fagocíticas estimulando diversas alterações na mucosa intestinal, com a liberação de citocinas pró-inflamatórias que resultam na formação da diarréia aguda.

Os mecanismos de patogenicidade de *S. enterica* estão relacionados com os genes codificados pelas ilhas de patogenicidade (PAIs), ilhas genômicas de *Salmonella* (SGI), plasmídeos de virulência, transposons, vírus lisogênicos e integrons, que são importantes para a invasão, sobrevivência dentro das vesículas denominadas de *Salmonella-containing Vacuole* (SCV), replicação e fuga do sistema imune do hospedeiro. Dentre eles, os fatores produzidos por estes conjuntos gênicos, incluem o sistema de secreção do tipo 3 (T3SS), o sistema de comunicação celular por *quorum sensing* (QS), várias proteínas efetoras e numerosos fatores de resistência.

O controle de *S. enterica*, especificamente no âmbito hospitalar, é realizado com a intervenção de antibióticos. Essa prática tem gerado grande preocupação na atualidade em virtude da crescente seleção de estirpes resistentes. Assim, estratégias alternativas para o controle de *S. enterica* são demandadas.

O controle do crescimento microbiano utilizando peptídeos antimicrobianos tem sido utilizado ao longo dos anos. Dentre eles, destaca-se o lantibiótico nisina, um peptídeo catiônico, que tem como alvo o lipídeo II da membrana interna dos procariotos. Trata-se do único peptídeo aprovado pelos órgãos de saúde e alimentos dos Estados Unidos e pela legislação brasileira para aplicação como conservante em alimentos.

A pouca eficácia da nisina na inibição do crescimento de bactérias Gram-negativas, como *Salmonella* spp., é amplamente demonstrado na literatura científica. A razão da resistência desse patógeno à nisina é relacionada à presença da membrana externa, que atua como uma barreira de proteção na superfície bacteriana, impedindo o acesso da nisina ao seu sítio alvo. Contudo, até o momento, não há relatos das possíveis modulações fenotípicas e fisiológicas que a nisina pode estimular em *S. enterica*.

Considerando o atual estádio de desenvolvimento do conhecimento da regulação de virulência de *Salmonella*, o objetivo deste trabalho foi o de analisar a modulação da virulência, as respostas a diversos tipos de estresses e a expressão gênica de *S. enterica* na presença de nisina.

MINI REVIEW: RECENT POTENTIAL APPLICATIONS OF NISIN AS A SIGNALING MOLECULE

ABSTRACT

Nisin is a bacteriocin belongs to lantibiotics group, synthesized ribosomal by some lactic acid bacteria (LAB) with antimicrobial activity against several types of pathogenic bacteria, as well as those resistant to antibiotics. Since its approval as safe for human consumption, nisin has been used as a food preservative in the control of various bacterial groups. However, in recent years, there has been a growing interest in expanding nisin applications that have surpassed the food context. Thus, in this mini-review, we explore the function of nisin as a signaling molecule of the bacterial response and the importance of this function in the microbial ecological context.

1. INTRODUCTION

Bacteriocins comprise a family of peptides that are synthesized ribosomally by some of LAB and are distinguished by their antimicrobial activity against several types of pathogenic bacteria, including those that are resistant to antibiotics (McAuliffe et al., 2001; Perez et al., 2015). Class I bacteriocins are known as lantibiotics, so named because of the presence of unusual amino acids, lanthionine and methionanethionine and their structure (Chatterjee et al., 2005). Among the lantibiotics of this class, it stands out nisin, a small peptide that interacts with lipid II and acts on the inhibition of cell wall synthesis and on the formation of pores in the membrane (Punyauppa-path et al., 2015; Juturu and Wu, 2018).

The characterization of the nisin molecule was first performed by Mattick and Hirsch (1947), who called it "N-inhibitory substance". Further research on nisin was intensified (Frazer et al., 1962; Hara et al., 1962; Shtenberg, 1973; Hurst, 1981) and after the results of the toxicity tests demonstrated their safety for human consumption ("Substance Generally Recognized as Safe" - GRAS), nisin was approved for use in food in 1988 by the Joint Food and Agriculture Organization / World Health Organization (FAO / WHO) (Deegan et al., 2006; Sobrino-Lópes et al., 2008).

Since its approval, nisin has been used as a food preservative in the control of various bacterial groups (Matamouros and Miller, 2015; Field et al., 2015, 2016a, b). A recent literature reviews of the role of nisin has revealed that there has been a great deal of interest in expand nisin use, in addition to the context of the food industry, which encompasses the medical context such as spermicidal agent, anticancer and antiplaque (Reddy et al., 2004; Tanphaichitr et al., 2016; Ahmad et al., 2017; Baindara et al., 2018; Mitra et al., 2019). Indeed, ecological applications, as a marker of responses in the natural niche, such as biofilm formation, QS, bacterial repellency, and gene expression regulator are also proposed (Horinouchi et al., 2010; Zhao et al., 2016; Drider et al., 2016; Chikindas et al., 2018). Therefore, in this mini-review, we will explore the functions of nisin as a signaling molecule of the microbial response and perform a prospect of the importance of this secondary function in the microbial ecological context.

2. BIOSYNTHESIS AND REGULATION

Biosynthesis of nisin through LAB fermentation involves the transcription of a cluster of 11 chromosomal genes annotated as *nis*ABTCIPRKFEG and organized into four transcriptional units, where the *nis*A gene is a structural gene encoding the precursor peptide of nisin A (consisting of 57 amino acids - aa), the *nis*BTCP operon, which acts on the dehydration, cycling and translocation of the prepeptide, the *nis*I gene, located internally in the *nis*BTCP operon, which encodes an immune response protein for binding, the *nis*RK genes, which comprise a regulatory system of two components and *nis*FEG operon, which encodes the immunity proteins for remoteness. Transcription of this gene cluster is regulated by three promoters called *PnisA* regulates transcription of this gene cluster, *PnisR* and *PnisF*, in *L. lactis* (Figure 1) (Tolonen, 2004; Lubelski et al., 2008; Ni et al., 2017).

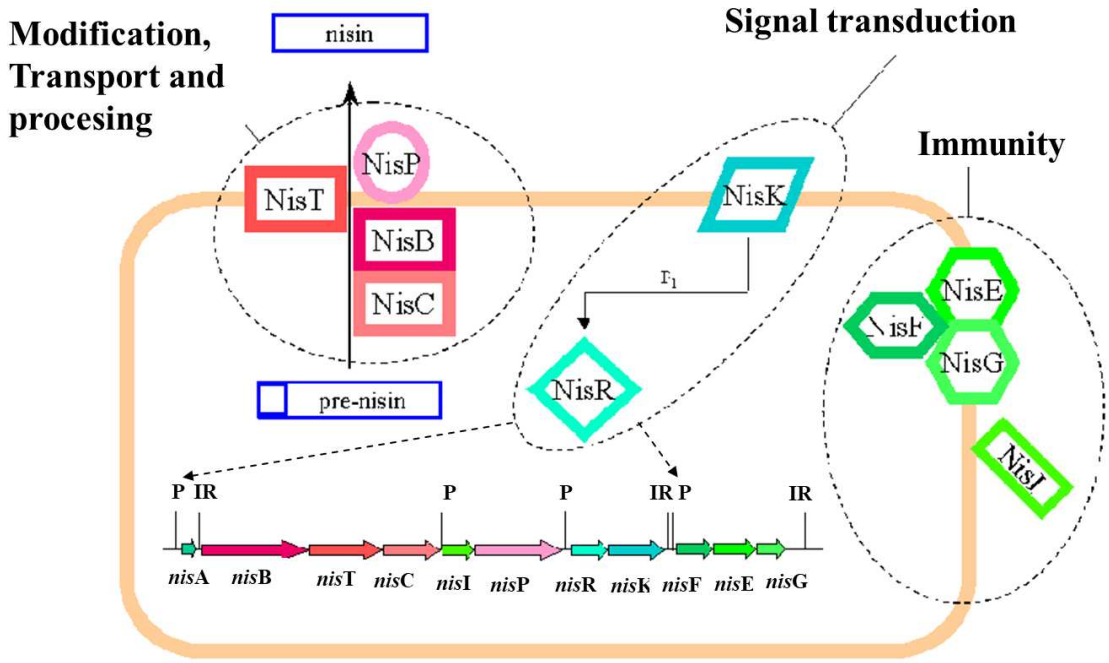

Figure 1: Model for regulation of nisin production in *Lactococcus lactis* by fermentation. Adapted from Tolonen (2004) and Perez et al. (2015).

The sequence of events for the production of active peptides begins with the expression of the structural *nis*A gene, which is modified by the NisB and NisC proteins, which dehydrate and cyclize the pre-peptide, which is translocated into the extracellular space, by the NisT transporter (Perez et al., 2015).

Then, the NisP protease recognizes the N-terminal leader peptide (23 aa) cleaving it to release the peptide nisin A (34 aa), in its active form, which accumulates in the extracellular medium, where it can act against neighboring bacteria, as well how to act as a QS signaling molecule (Vuyst and Vandamme, 2012; Ni et al., 2017).

It is known that nisin production is controlled by QS, a bacterial communication system that is directly related to the regulation of genes in response to population density. This signaling is linked to the accumulation of autoinducing molecules, synthesized and

diffused freely through the membrane, to the external environment. The perception of these molecules occurs when autoinducer concentrations become critical in the external environment, detected by sensor proteins also called R proteins, which trigger the regulatory response over gene expression (Reuter et al., 2016).

Positive regulation of nisin biosynthesis is controlled by NisK, a kinase protein located on the cell membrane and the NisR regulator. For this, the NisK protein is induced by nisin A, acting as a signal-inducing molecule as it accumulates outside the cell (Horinouchi et al., 2010) which is phosphorylated and, as a cascade effect, phosphorylation of NisR protein which activates the promoters PnisA and PnisF (Perez et al., 2015; Ge et al., 2017).

Interestingly, nisin-producing bacteria possess a mechanism of resistance to the action of the same, which is carried out by NisI lipoprotein. In this case, this protein binds to nisin A located in the around of the producing cell, providing protection against antimicrobial action. There is also the action of the NisFEG complex in this mechanism, which provides additional protection to the producer cells, expelling the nisin A, away from the cell (Perez et al., 2015).

3. NISIN STRUCTURE

Nisin belongs a class I bacteriocin named lantibiotic, defined as a small linear cationic peptide (3.5 kDa), which contains unusual amino acids, modified after the translation as lanthionine or β-methyl-lanthionine, as well as numerous dehydrated amino acids. Nisin is ribosomally synthesized as an inactive propeptide consists of the 57 amino acids. After translocation to the extracellular space undergoes cleavage by the NisT protease, which releases the active form of the peptide which is composed of 34 amino

acids of which 13 are unusual, and no aromatic amino acid residues, make the overall structure of nisin unique. The interaction of these unusual amino acids with the other amino acids results in a polycyclic structure, with five rings (A, B, C, D and E, Figure 2) (Manzor et al., 2017), which are essential for the maintenance of stiffness, resistant to proteolytic degradation and thermal inactivation (McAuliffe et al., 2001; Perez et al., 2015). In addition, the specific presence of the A and B rings of the N-terminus of nisin (nisin 3 - 12) are critical to its overall biological activity where it binds to the lipid II moiety inhibiting bacterial cell wall biosynthesis. To date, five naturally occurring variants of nisin A, Z, Q, F and, U, differ structurally in amino acid residue types, but with similar activity, and nisin A is the most extensively studied bacteriocin (Horinouchi et al., 2010; Piper et al., 2011; O'Connor et al., 2015; Gharsallaoui et al., 2016a).

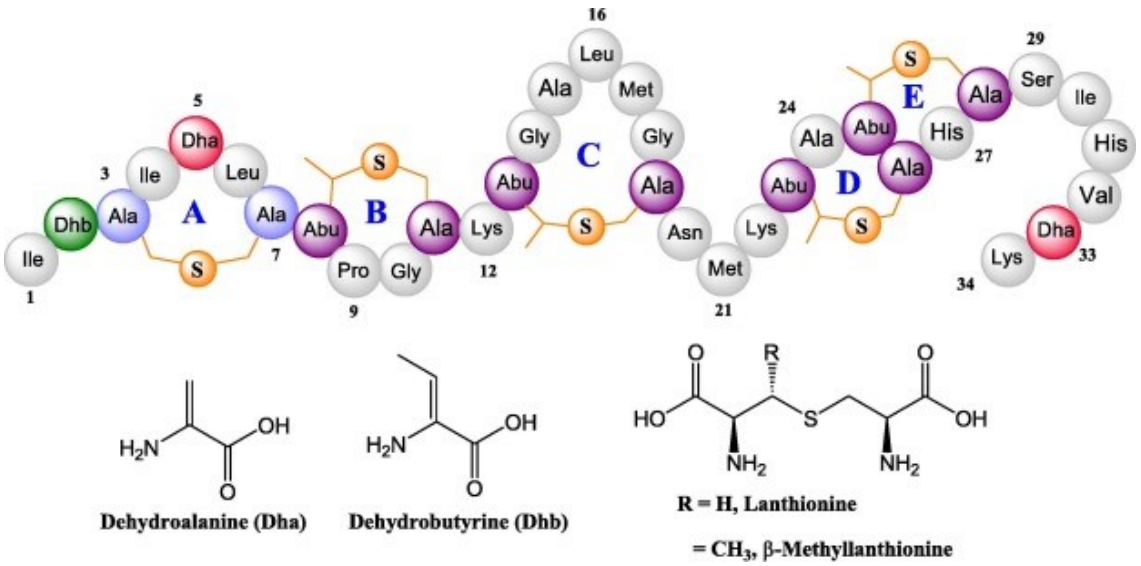

Figure 2: The amino acid sequence of nisin A and the structures of its unusual amino acid residues and five internal rings (A–E) within its overall sequence. The unusual amino acids present in nisin are dehydroalanine (Dha), dehydrobutyrine (Dhb), lanthionine or b-methyllanthionine. The A-ring contains a lanthionine bridge (Ala-S-Ala) while the remaining B-E rings contain b-methyllanthionine bridges (Abu-S-Ala). Adapted from Manzor et al. (2017).

4. MECHANISMS OF ACTION

The mechanism of action of nisin in Gram-positive bacteria involves the inhibition of cell wall synthesis by the interaction with lipid II, which is a precursor of peptidoglycan biosynthesis, membrane pore formation, dissipation of lipid II in cytoplasmic membrane, and other changes due to these modulations, resulting in bacterial death (Figure 3) (Cotter et al., 2005; Hasper et al., 2006; de Kruijff et al., 2008).

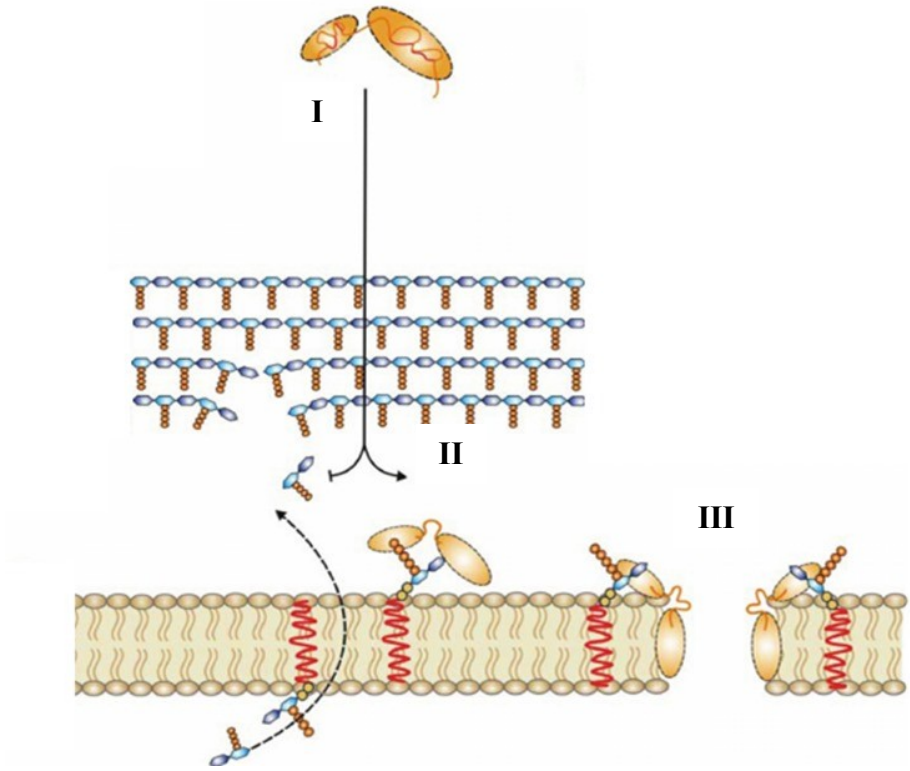

Figure 3: Mode of action of nisin as bacteriocin. I- Nisin in the extracellular medium. II- Inhibition of cell wall synthesis. III- Formation of pores in the membrane. Adapted by Cotter et al. (2005).

Initially, an event occurs between nisin and the surface of the target cell, which consists of the adsorption for the destabilization of the cytoplasmic membrane. This adsorption involves electrostatic interactions between nisin with a net positive charge and negatively charged membrane phospholipids (Cotter et al., 2005). Under these conditions,

nisin binds to lipid II pyrophosphate with the two lanthionine rings located in the N-terminal region, leading to pore formation in the membrane, which involves a stable transmembrane orientation of nisin (Hsu et al., 2004; Medeiros-Silva et al., 2019). The effectiveness of this integration depends on the nature and content of the cell membrane phospholipids, which may explain differences in sensitivity between target bacterial strains (Gharsallaoui et al., 2016a). The insertion of the C-terminal region, which until then was transmembrane, is facilitated by the flexible region of nisin, which eventually forms complex and leads to stable pores formation, according to the number of nisin inserted (tetramer or pentamer) (Breukink and Kruijff, 2006; Prince et al., 2016).

The mechanism of action of nisin that involves the destabilization of the cytoplasmic membrane generates the formation of small pores in the membrane with the release of ions, ATP, potassium ion, amino acids and other small molecules. The release of these essential components of the cytoplasm and, or cellular lysis, results in the death of bacteria (Gharsallaoui et al., 2016a). An alternative mechanism of bactericidal action of nisin involves the dissipation of lipid II and nisin-induced lipid II sequestration in large domains as a result of the interaction of high affinity between this lantibiotic and its receptor on membranes (Cotter et al., 2005; Paiva et al., 2011).

In Gram-negative bacteria, the outer membrane acts as a protective barrier and nisin is effective against this bacterial group only in association with different growth conditions and permeabilizing substances (Matamouros and Miller, 2015; Prudêncio et al., 2015). Some studies report the synergistic effect of nisin in the presence of ethylenediamine tetraacetic acid (EDTA), citrate, lysozyme, antibiotics, plant essential oils, others antimicrobial peptides, low pH, among others, as well as the use of nisin variants and nisin nano-engineered (Bader et al., 2003; Prudêncio et al., 2015; Galvão et

al., 2015; Field et al., 2012; 2015; 2016a; 2016b; Singh et al., 2013; Vukomanović et al., 2017).

5. NISIN: AS SIGNAL MOLECULE

The responses induced by the presence of nisin are more easily observed when the bacteria are exposed to low concentrations of bacteriocin, levels usually found in the natural environment (Takhistov et al., 2009). In this condition, it is possible to discuss several mechanisms activated by nisin, such as the induction or inhibition of QS (Horinouchi et al., 2010), the signaling to repel bacteria (Chikindas et al., 2018), the regulation of gene expression (Zhao et al., 2016; Pimentel-Filho et al., 2014), induction resistance to nisin (Perez et al., 2014), and reduced cell adhesion (Pimentel-Filho et al., 2014), which are different from the bactericidal action, observed markedly when there are high concentrations of nisin (Takhistov et al., 2009).

5.1 *Signaling of quorum-sensing*

Gram-positive bacteria produce quorum Sensor Peptides (QSPs) or Self Inducing Peptides (AIPs) that act as a signaling molecule to mediate specific cellular response. Nisin exerts dual function, acting both on QS signaling and on microbial growth control (Sturme et al., 2002; Wynendaele et al., 2012; Kareb and Aïder, 2019). In regulating the QS, nisin is expressed at basal levels and remains inactive in the cytoplasm, whereafter processing and secretion, accumulates outside the cell, until it reaches a threshold concentration, dependent on cell density, to induce the QS (Figure 4) (Mascher et al., 2006; Keller and Surette, 2006; Roy et al., 2011).

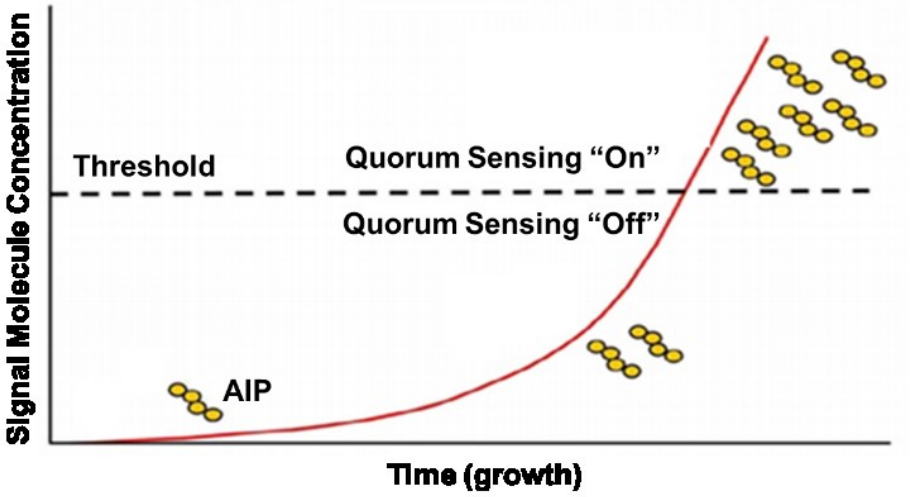

Figure 4: Schematic representation of the triggering of a system QS *Lactococcus lactis*. Adapted from the review of Roy et al. (2011).

To induce quantum sensing, nisin uses the NisK / R two-component system of *L. lactis*. Initially, nisin binds to the NisK membrane protein recognition site, a histidine kinase, which undergoes autophosphorylation (ADP / ATP), transferring a phosphate group to its cognate response regulator, the cytoplasmic protein NisR, which in turn activates the expression of the structural and regulatory genes of nisin, leading to differential expression of these genes (Kleerebezem et al., 1997; Lubelski et al., 2008). Considering that AIP, like nisin, is detected by the surrounding organisms with the same signaling system, this mechanism leads to an explosion in the release of active nisin in a synchronized way (Figure 5) (Hvarstein, 2003; Mascher et al., 2006; Choudhary and Schmidt-Dannert, 2010).

It is important to emphasize that during this process, the structures of rings A and B of the N-terminal region of nisin are essential for the activation of the NisK protein, and leucine in position six deserves emphasis for this activity (Ge et al., 2016). Some hydrophobic residues conserved in the extracellular (C-terminal) region of the NisK

protein are also important for the detection of nisin and regulation of its biosynthesis (Ge et al., 2017).

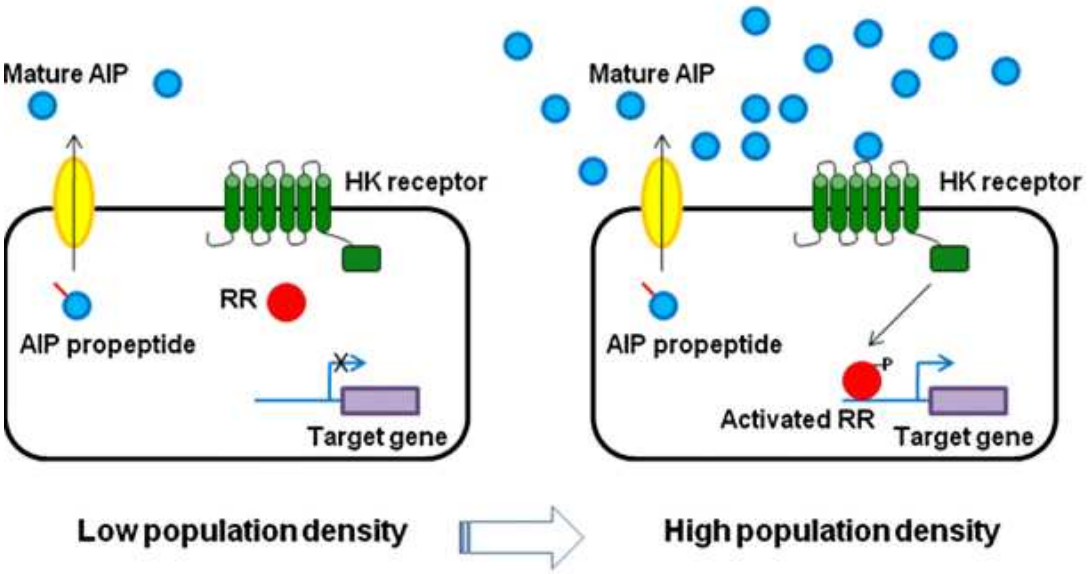

Figure 5: Extracellular detection of the autoinducing peptide (AIP) by the NisK / R two-component system. The accumulation of AIP in the extracellular environment is a threshold for the induction of the HK membrane protein (green), which in turn phosphorylates the RR effector (red), with the activation of the expression of the specific genes. Adapted from Choudhary and Schmidt-Dannert (2010).

Alternative way of nisin biosynthesis regulation was described with nisin Z, a variant of nisin A. In this case, it was observed that when secretion of nisin Z was blocked, intracellular proteolytic activity cleaved the N-terminal leader peptide from the precursor of nisin, resulting in accumulation of the active peptide in the cytoplasm, which interestingly activated the signal transduction of NisK by extruding parts of the nisin molecule into the pseudoperiplasmic space, where it was able to interact with the histidine kinase signal recognition domain (Hilmi et al., 2006).

5.2 *Influence on gene expression*

Low concentration of nisin was able to affect the expression of some genes in *Staphylococcus aureus* and a significant increase of *icaD* and *fnbA* genes, related to adhesion and biofilm formation was observed (Pimentel-Filho et al., 2014). Shin et al. (2016) presented information about the importance of nisin, not only as an anti-biofilm compound but also, as defense peptides for the host, being able to induce the adaptive immune response and play an immunomodulatory role. It has also been mentioned that low nisin concentrations can modulate the expression of more than 600 genes in *S. aureus*, among them genes associated with pathogenicity factors, adaptations to atypical conditions, lipid metabolism, RNA synthesis (Zhao et al., 2016). However, it is not clear how this mechanism occurs.

5.3 *Inhibition of the biofilm formation*

Biofilm is a resistance phenotype that bacteria exhibit to outperform adverse conditions, such as exposure to antimicrobials (Mah and O'Toole, 2001). The resistance conferred is due to the presence of the protective barrier that the biofilm matrix provides, reducing the entry of antimicrobials into the cell (Mah and O'Toole, 2001), and also by repelling or capturing antimicrobials through exopolysaccharides and capsular polysaccharides (Vuong et al., 2004). Highlighting the potential of antimicrobial peptides against formed biofilms as a current alternative and the effectiveness of nisin, against bacterial biofilms have been evaluated (Mahdavi et al., 2007; Coughlan et al., 2016; Mathur et al., 2018; Kim et al., 2019). However, few studies focused on the effect of low nisin concentration on inhibition of steps of biofilm formation. Using subinhibitory nisin dosage, Pimentel-Filho et al. (2014) showed that the bacteriocin in synthetic medium reduced the *S. aureus* adhesion to the polystyrene surface.

Low concentrations of nisin inhibited planktonic growth of oral bacteria and retarded the development of multi-species biofilms, reducing biofilm biomass and thickness in a dose-dependent manner (Shing et al., 2015). These authors also observed that the biofilm formed in the presence of nisin was highly disintegrated and lacked coaggregative behavior.

5.4 *Modulation of the bacterial surface*

Changes in bacterial surface charges are used by Gram-positive and Gram-negative bacteria to rule out or prevent the interaction of antimicrobial peptides at the target site, giving a resistance response (Joo et al., 2016). Mechanisms of resistance to antimicrobial peptides in bacteria are well understood in the literature and may occur by surface remodeling resulting in charge modification, increased membrane hydrophobicity, to reduce the attraction and insertion of antimicrobial peptides at their target site (Ernst et al., 2011; Kumariya et al., 2015*;* Matamouros and Miller, 2015).

An observing mechanism in both, Gram-positive and Gram-negative bacteria, is the aminoacylation of phosphatidylglycerol with lysine, which results in the change of lipid head loads with the electrostatic repulsion of the antimicrobial peptide (Ernst et al., 2015). In the teichoic acids, a process called D-alanylation, which gives a higher positive charge to these structures, also occurs, removing antimicrobials such as nisin (Peschel et al., 1999).

Palminoylation of lipopolysaccharide (LPS) and also of the membrane phosphatidylglycerol is common, through the action of the PagP protein, which is involved in the membrane hydrophobicity homeostasis. It is also suggested that it performs the repair in membranes damaged by antimicrobial peptides (Band and David, 2015). In Gram-negative bacteria, such as *Salmonella* Typhimurium, the two-component

systems PhoPQ and PmrAB, which regulate the lipid A modification of the outer membrane LPS, making it more rigid and changing the surface charge of the bacterium to repel antimicrobial peptides (Choi et al., 2009; Mascher et al., 2006; Dalebroux et al., 2014). It is described that the PhoQ kinase sensor is activated when there is an increase in antimicrobial peptide concentrations, a mechanism commonly encountered during macrophage invasion (Band and David, 2015; Goto et al., 2017).

Thus, it seems that nisin is also capable of triggering these mechanisms in bacteria within a microbial community, since it has been demonstrated the expression of genes for the regulation of these resistance systems in the presence of cationic antimicrobial peptides similar to nisin in *Salmonella* Typhimurium (Bader et al., 2003) and *S. aureus* (Zhao et al., 2016).

5.5 *Ecological importance*

The alteration of the intestinal microbiota mediated by bacteriocins produced by resident bacteria can be considered to understand the importance of nisin activity in the dynamics of the gastrointestinal microbiota (Donia et al., 2014; 2015). The presence of several types of molecules, including bacteriocins, has been demonstrated and is expressed by commensal bacteria in the gastrointestinal tract (Kommineni et al., 2015; Guinane et al., 2016). These peptides impact niche competition by making bacterial therapeutic compensation, precisely eliminating pathogenic bacteria, without causing an imbalance in the intestinal microbiota, demonstrating the ecological relationship between these bacteria (Kommineni et al., 2015; Guinane et al., 2016; Drider et al., 2016).

Observing the dynamics between nisin-producer and non-producer bacteria grown in co-cultures, specifically in presence of *Listeria monocytogenes* and *S. enterica*, it was possible to understand that pathogenic bacteria affect the expression of nisin genes and consequently, their synthesis by producing bacteria, leading us to believe that there

is a recognition when these microorganisms are kept in the same environment, it can result in an antagonistic response by competition of niches (Abdollahi et al., 2018).

6. CONCLUSION

Nisin is a peptide capable of inducing various response mechanisms within a microbial community where the peptide-producing bacteria are found. These results indicate that, under sublethal conditions, the peptide acts as a stressor, stimulator or inducer of different bacterial phenotypes such as gene expression, resistance, biofilm formation, quorum sensing.

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CHAPTER 2

STRESSFUL CONDITION ENHANCES THE EFFECT OF NISIN AGAINST *Salmonella enterica*

ABSTRACT

Salmonella enterica, a Gram-negative pathogen of global importance, was recognized by the ability to trigger adaptive responses under specific stressful conditions. As a Gram-negative bacterium, this pathogen is more resistant to the bacteriocin nisin due to its outer membrane, which acts as an effective barrier. However, physical and chemical treatments can improve the action of nisin against *Salmonella*. The objective of this study was to verify the responses of *S. enterica* to the presence of nisin combined with stressing agents. Sub-MIC concentrations of 11.72 μM, 23.44 μM and, 46.88 μM nisin were choice to evaluate the effect of bacteriocin against *S. enterica*. The percentage of cell death in the presence of nisin was higher ($p < 0.05$) in the first hour of incubation and decreased in 9 hours. Biofilm formation was significantly affected by concentrations of nisin above 46.88 μM. Sub-lethal stress conditions such as low pH, 4.0-5.5, NaCl up to 5% and H_2O_2 concentrations of 500 and 1000 μM significantly influenced the growth of *S. enterica* and, cells under pH and NaCl stress were more sensitive to inhibition by nisin. This increased sensitization to nisin was also significant in the presence of antibiotics that inhibit cell wall biosynthesis as ceftriaxone, meropenem, cephalexin and ampicillin, and DNA replication, as norfloxacin. The sub-MIC concentration of nisin did not alter the expression of *glgC* and *fliF* genes related to biofilm formation after 1 and 6 hours of treatment. However, *fimF* which acts on biofilm formation was differentially expressed after 1 hour of treatment with nisin sub-MIC. In conclusion, membrane destabilizing agents such as low pH, high concentrations of sodium chloride and antibiotics enhanced the inhibitory activity of nisin on *S. enterica*.

1. INTRODUCTION

Salmonella enterica is a Gram-negative pathogen that causes gastroenteritis and enteric fevers of global importance (Card et al., 2016). This pathogen is associated with one of the important zoonoses that worried the public health and is responsible for economic losses in the food industry, especially in the poultry industry (Doyle et al., 2015). According to the Center for Disease Control and Prevention (CDC), salmonellosis is estimated to cause more than 1.2 million illnesses each year in the United States, with more than 23,000 hospitalizations and 450 deaths (CDC, 2016). Worldwide, *Salmonella* is among the top four causes of diarrheal disease from unsafe food, affecting at least 550 million people each year, of these, approximately 220 million of which are children under 5 years of age (WHO, 2017).

Epidemiological data from the Ministry of Health show that *Salmonella* was responsible for 35% of outbreaks of foodborne diseases in Brazil, registered in the period from 2000 to 2017 (Brasil, 2018). These data correspond mainly to the ingestion of eggs and dairy products, consumed in residences and restaurants/bakeries, especially contaminated by phagotype 4 (Santos et al., 2003; Nunes et al., 2003; 2013; Brasil, 2018).

The pathogenic ability of *Salmonella* is associated with genes present on the pathogenicity islands (SPI) *Salmonella* genomic islands (SGI), virulence plasmids, transposons, lysogenic and integrons viruses are essential for invasion, survival, replication, and escape of the host immune system (Wisner et al., 2012). Also, *S. enterica* triggers adaptive responses under specific stressful conditions, such as low pH and temperature extremes, presence of reactive nitrogen and oxygen species, nutrient limitation, oxygen tension, bile salts, free fatty acids, and antimicrobial peptides, redirecting appropriate gene expression (Fang et al., 2016). This adaptation may occur through the presence of the specific proteins that detect the environmental signal and

initiates a signaling cascade in response to stressful conditions (Alvaréz-Ordonez et al. 2015).

The increase in antibiotics resistance, mainly against β-lactams as penicillins, cephalosporins, carbapenems, and monobactams, and also against aminoglycosides, as streptomycin, conferring advantages for *Salmonella* to remain in the environment (Singh et al., 2014). Besides the high resistance to stress conditions and antibiotics, other factors contribute to the permanence of *S. enterica* in the environment, among them, the ability to form a biofilm (Finn et al., 2013). Biofilm can be considered a microbial life-style in which microorganisms of the same or different species are incorporated into an extracellular polymeric matrix (EPS) exhibiting altered physiological characteristics, compared with their planktonic homologous cells (Donlan and Costerton, 2002; Vogeleer et al., 2014). The process of formation of *S. enterica* biofilm is well known, highly regulated (Simm et al., 2014; Vadyvalooand Martínez, 2014) and offers advantages for this pathogen, leaving it with higher resistance to temperatures, pH, desiccation, ultraviolet radiation, sanitizing and antimicrobial-mediated killing (Annous et al., 2009; Yang et al., 2016).

The control of bacterial infections is realized with antibiotics, but the current scenario of bacterial resistance has generated worries and, new control alternatives have been investigated (Crofts et al., 2017). The bacteriocins, among them nisin, have been comprehended as options to inhibit pathogens in association with antibiotics (Singh et al., 2013; Rishi et al., 2018). It is believed that the synergistic effect among nisin and antibiotics observed against Gram-positive bacteria as *Enterococcus faecalis* (Tong et al., 2014) and *Staphylococcus aureus* (Brumfitt et al., 2002) and against Gram-negative as *S. enterica* serovar Typhimurium is related to membrane permeabilization (Singh et al., 2014).

Nisin is a cationic antimicrobial peptide (AMPs) targeted to lipid II of the inner membrane of prokaryotes and, has been intensively used in the microbial control as a food preservative approved in several countries (Hasper et al., 2006; Zhao et al., 2016). It is recognized as "Generally Regarded As Safe" (GRAS) and by food agencies of the United States and by the Brazilian legislation for use as a food preservative (Brasil, 1996; Joint, FAO/WHO, 2014). Nisin alone has low efficacy against Gram-negative pathogens, due to the presence of the outer membrane (Matamouros and Miller, 2015; Prudêncio et al., 2015). Despite this, the use of nisin combined with acid, temperature, ethylenediamine tetraacetic acid (EDTA), antibiotics and anti-Gram-negative peptides has been explored (Bader et al., 2003; Galvão et al., 2015; Matamouros and Miller, 2015; Prudêncio et al., 2016).

Although nisin has shown a low efficiency in *S. enterica* growth, it is not clear whether this bacteriocin modulates the phenotypes in this pathogen. The ability of nisin on gene modulation was demonstrated in Gram-positive bacteria, such as *S. aureus*, with increased expression of genes associated with biofilm formation (Pimentel-Filho et al., 2015) and 22 categories of differentially regulated genes related with stress response, virulence factors and, biofilm formation (Zhao et al., 2016). Although gene modulation in nisin-treated *Salmonella* has not been demonstrated, it is known that cationic AMPs, such as polymyxin, is capable of altering the transcription of different classes of genes in *Salmonella* Typhimurium, such those related with biofilm formation, virulence, flagellum and stress resistance (Bader et al., 2003)

Some studies have analyzed the effect of nisin as a signaling molecule or inducer of the microbial phenotype response (Horinouchi et al., 2010; Zhao et al., 2016; Drider et al., 2016; Chikindas et al., 2018). This approach is based on the idea that in the natural environment, biosynthesis of bacteriocins is regulated and dependent on the ecological

niche conditions in which the bacterium is located (Donia et al., 2014; 2015). These mechanisms may be better observed under conditions of low concentrations of nisin available in the medium (Chikindas et al., 2018). Considering this insight, the aim of this work was to analyze the responses of *S. enterica* to the presence of nisin combined with stressing agents commonly found in foods or food industry environment as low pH, sodium chloride and, hydrogen peroxide.

2. MATERIALS AND METHODS

2.1 Bacterial strain

S. enterica serovar Enteritidis phage type 4 (PT4) 578, isolated from chicken meat, provided by Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Brazil), was used in this study and has been previously described (Campos-Galvão et al., 2015). The stocks culture was performed in brain and heart infusion broth (BHI, Oxoid, England), supplemented with 20% (v/v) sterilized glycerol and stored at -20 °C. Before the experiments, the bacterial cultures were reactivated in BHI for 24 hours at 37 ± 1 ° C aerobically. After growth, cells were centrifuged at 6,000 *g* for 10 minutes, washed twice in 0.85% saline. The inoculum was standardized to 0.1 of optical density at 600 nm (OD_{600nm}) (approximately 10^7 CFU / mL) using a spectrophotometer (Thermo Fisher Scientific, Finland).

2.2 Preparation of the nisin solution

The stock solution of nisin was prepared by dilution of Nisaplin (2.5% Nisin, Nisaplin®, kindly provided by Danisco Brazil) in 5 mL of sodium phosphate solution (5 mM, pH 2.0). The final stock concentration was 1,500 μM. After preparation, the stock solution was stored under refrigeration until used in the experiments.

2.3 The minimal sub-inhibitory concentration of nisin (sub-MIC)

The sub-inhibitory concentration of nisin was determined to monitor the growth of *S. enterica* in BHI broth supplemented with different concentrations of nisin (2.93 to 185.50 μM). Aliquots of 200 μL of broth were transferred to 96-well polystyrene microplates and inoculated with approximately 10^7 CFU / mL of the bacterial culture previously propagated in the media BHI. The microplate was incubated in a microplate reader (Thermo Scientific, Multiskan GO UV / Vis, Waltham, UK) at 37 °C for 12 hours. Optical density (OD_{600nm}) readings were performed every 15 minutes, and the values obtained were used to construct the growth curves of the pathogen in the different nisin concentrations. The experiments were performed in triplicates and with two biological replicates. The minimum concentration that does not interfere with the growth of *S. enterica* during the 12 hours was used as the sub-MIC in this work. Growth in BHI without nisin was used as an experimental control.

2.4 Analysis of cell viability in the presence of sub-inhibitory concentrations of nisin

The viability of *S. enterica* was analyzed in the presence of sub-inhibitory concentrations of nisin on the flow cytometer (BD Bioscience, Calif., USA). Cells in the exponential phase were centrifuged at 6,000 *g* for 10 minutes and washed twice with saline and standardized to a concentration of approximately 10^6 CFU / mL. Propidium iodide (PI) was then added for analysis of cell viability. Viability determination was performed on the flow cytometer every one hour for nine hours. Cells grow in media without nisin were used as controls.

2.5 Biofilm formation

Biofilm formation by *S. enterica* was evaluated under different sub-inhibitory concentrations of nisin using the plates previously prepared for growth assessment,

according to a protocol adapted from Almeida et al. (2017). Twenty-four hours after incubation, the culture media was discarded, the wells washed with sterile distilled water and cells adhered to the surface stained for 30 minutes with 200 µL of 0.1% (w / v) crystal violet. Subsequently, the crystal violet was removed, and the wells were washed three times with distilled water. The plates were dried for 15 minutes in an oven at 40 °C. Then, 200 µL of 95% alcohol (v / v) was added to each well and the OD of the crystal violet extract was determined on a microplate reader at wavelength 590 nm (OD_{590nm}). Data were expressed as the ratio between the absorbance of crystal violet extract of adhered cells and the optical density of total cells (OD_{600nm}/OD_{590nm}) (Viana et al., 2009).

2.6 *Salmonella* tolerance to stress conditions in the presence of the sub-inhibitory concentration of nisin

Aliquots of 200 µL of BHI broth (pH 6.8) inoculated with exponentially growing *S. enterica* cells were distributed into 96-well microplates and exposed to different stress conditions combined with a sub-inhibitory concentration of nisin (11.72 µM). The stress conditions were low pH (4.5 to 6.8), adjusted with hydrochloric acid; sodium chloride at concentrations varying from 0 to 5% and hydrogen peroxide at 500 to 1,000 µM. The microplates were incubated in a microplate reader at 37 °C for 24 hours. Optical density (OD_{600nm}) readings were performed every 15 minutes, and the values obtained were used to construct the growth curves of the pathogen in the different stress conditions. The experiments were performed in quadruplicate and with two biological replicates. Assay without nisin and different stress conditions were used as an experimental control.

2.7 The combined effect of nisin with antibiotics

The antibiogram combined with the sub-inhibitory concentration of nisin was carried out using antibiotic discs. A bacterial suspension was prepared in sterile saline

(0.85% NaCl) until turbidity compatible with the MacFarland scale grade 0.5 (10^6 CFU / mL) was obtained. The sub-inhibitory concentration of nisin was added in both bacterial inoculum and Mueller Hinton agar media. The culture was seeded in all directions of the agar using a swab and multi discs of antibiotics were added to the surface. An antibiogram plate was prepared without nisin. Plates were incubated at 37 ° C for 20 hours, and the inhibition halos were measured with the aid of a digital caliper.

2.8 Analysis of the gene expression of *S. enterica* treated with nisin

Cultures of *S. enterica* were reactivated in BHI broth incubated at 37 °C until reaching a population of 10^7 CFU / mL. Nisin, in sub-inhibitory and inhibitory concentrations, were added to culture media containing the bacterial inoculum in independent experiments. To the control culture, the same volume of PBS pH 2.0 was added. The cultures were incubated for 1 and 6 hours. After the incubation period, the cultures were centrifuged to obtain the total biomass and subsequent extraction of total RNA. Cells were homogenized with 600 µL of TRI Reagent® (Sigma-Aldrich, Canada) for 30 seconds in a vortex and incubated for 15 minutes at room temperature. Then, 200 µL of ice-cold chloroform were added followed by manual homogenization for 15 seconds, incubation for 2 minutes at room temperature and subsequent centrifugation at 12,000 *g*, at 4 °C for 15 minutes for separation of aqueous and organic phases. The aqueous phase was transferred to a new 1.5 mL microtube, added with 500 µL of isopropanol, incubated for 15 minutes at room temperature to precipitate the RNA and centrifuged at 12,000 *g*, 4 ° C for 10 minutes. The supernatants were discarded and 1 mL of ice-cold ethanol (75%) was added to the microtubes, homogenized and centrifuged at 7,500 *g*, 4 ° C for 10 minutes. The supernatants were discarded and the precipitated total RNA was allowed to air dry for the addition of RNase-free water for solubilization of the extracted RNA. The concentrations of the total RNA were evaluated by

spectrophotometer reading at 260 nm and 280 nm to calculate the 260/280 ratio. The integrity of the RNA was evaluated by electrophoresis in 1.5% (w/v) agarose gel. Total RNA was purified to eliminate the DNA with DNAse I (Promega, Madison, USA). From 1 μg of RNA, the syntheses of the first cDNA strands were performed according to the protocol recommended by the manufacturer of the ImProm- II™ reverse transcription system (Promega, Madison, USA). The RT-qPCR was performed in a reaction with a volume of 12 μL containing 1 μL of the cDNA template, 1 μL of the respective primers (Table 1) 6 μL of SYBR Green master mix and, 3 μL of sterile ultrapure water. Reactions were performed with the first step at 50 °C for 3 minutes, the second step of 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. All amplifications were performed on optical grids in 96-well plates in the Bio-Rad C1000 Manager sequence detector system. The fluorescence data were processed using the Bio-Rad CFX Manager software, resulting in cycle limit values for each sample. This experiment was performed with three independent replicates and each sample was evaluated in triplicate. The gene dosages were obtained by the 2-ΔΔCt method and the subunit of the DNA gyrase and 16S ribosomal was used as an endogenous control to correct the dosages of the genes of interest, and the control treatment was used as a target.

Table 1. Primers used in this study

Primer	sequences (5'–3')	Reference
*glgC*F	TGACCAGAACTGGCCTATCC	Campos-Galvão et al., 2015
*glgC*R	CAGCGAGTTCAGCGTCATAC	Campos-Galvão et al., 2015
*fimF*F	CCTAACACGTTACCGCTTCA	Campos-Galvão et al., 2015
*fimF*R	CCGGCGTCATATTTTCTGA	Campos-Galvão et al., 2015
*fliF*F	GAAGCCATTCTGTCGCCTAT	Campos-Galvão et al., 2015
*fliF*R	TGTAGTGCTCTTCCGTCTGC	Campos-Galvão et al., 2015
*gyrA*F	CCAATACGTTCATGGCGTAAAG	Campos-Galvão et al., 2015
*gyrA*R	GATTATGCGATGTCGGTCATTGT	Campos-Galvão et al., 2015
*16SrRNA*F	CGTGTTGTGAAATGTTGGGTTAA	Kollanoor Johny et al., 2017
*16SrRNA*R	CCGCTGGCAACAAAGGATAA	Kollanoor Johny et al., 2017

3 RESULTS AND DISCUSSION

3.1 Nisin interferes with the growth of *S. enterica*

Although nisin has a low effect upon Gram-negative bacteria, the growth of *S. enterica* was reduced ($p < 0.05$) in the presence of concentrations above 23.44 μM of this bacteriocin, equivalent to 78.60 μg of nisin / mL, according to the fabricant of Nisaplin® (Figure 1, A and B). This high resistance of *Salmonella* to nisin was related to

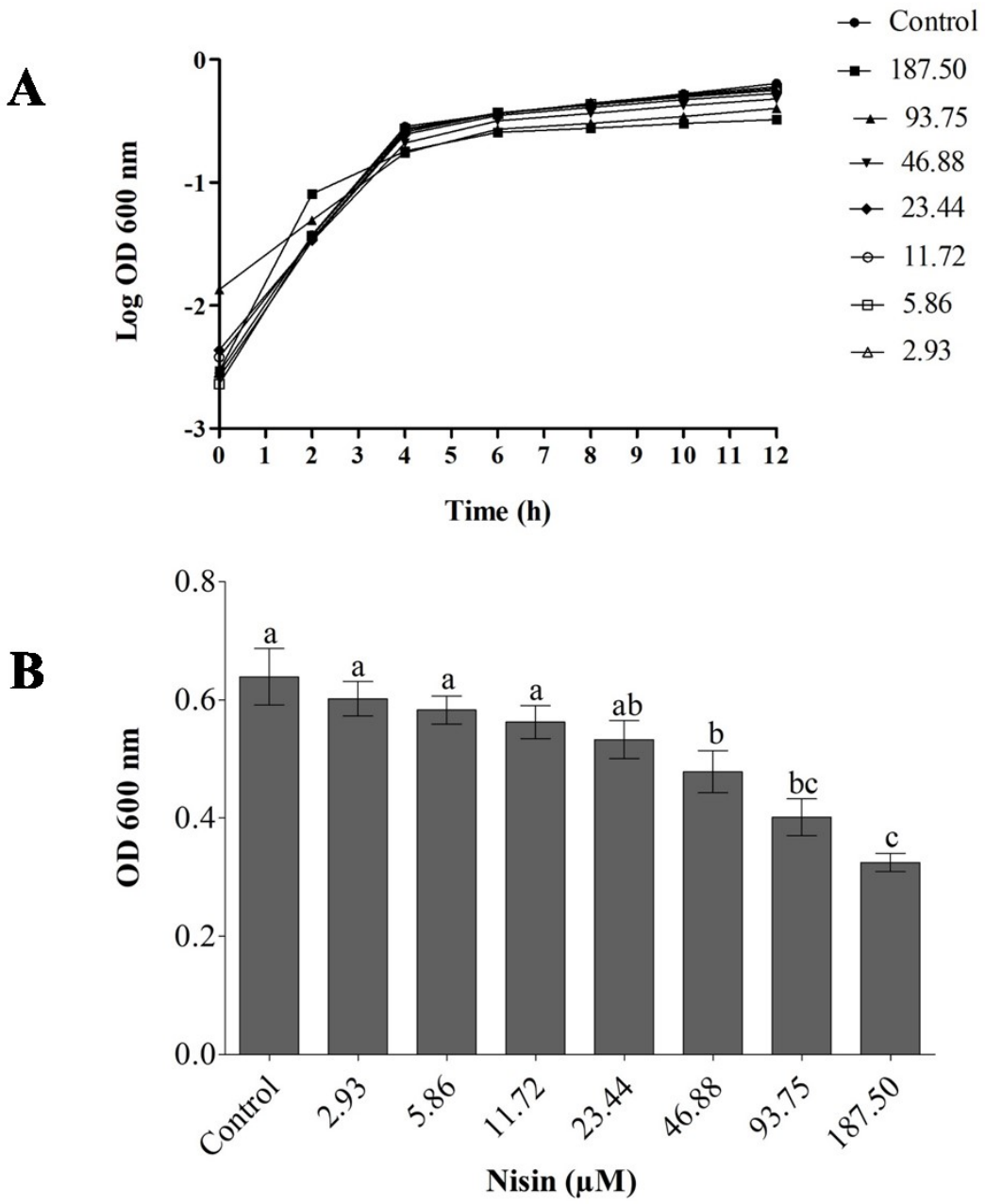

Figure 1: Growth curve of *S. enterica* in BHI broth under concentrations of nisin ranging from 0 to 187.5 µM along of 12-hour incubation (A), and after incubation period (B). Mean (*n=3*) and Standard error (± SEM) are shown (bars). Different letters indicate significant differences based on the Tukey HSD test ($p < 0.05$).

the presence of outer membrane that acts as an effective barrier and, according to Li et al. (2018), it is expected a high concentration of the bacteriocin to inhibit *Salmonella* growth. Lower concentrations of nisin could promote inhibition of the growth of *S. enterica* when it was associated with other factors, like low pH and high temperature (Galvão et al., 2015; Prudêncio et al., 2016); EDTA added in broth (Ukuku and Fett, 2004; Khan et al., 2015; Prudêncio et al., 2016; Sangcharoen et al., 2017; Bingol et al., 2018) or in Turkish-type meatballs (Bingol et al., 2018).

3.2 Nisin reduces cell viability of *S. enterica*

The percentage of cell death in the presence of nisin was higher that the control treatment ($p < 0.05$) in the first hour of incubation, with to 16.54% in the presence of 46.88 µM nisin and decrease over the 9 hours (Figure 2).

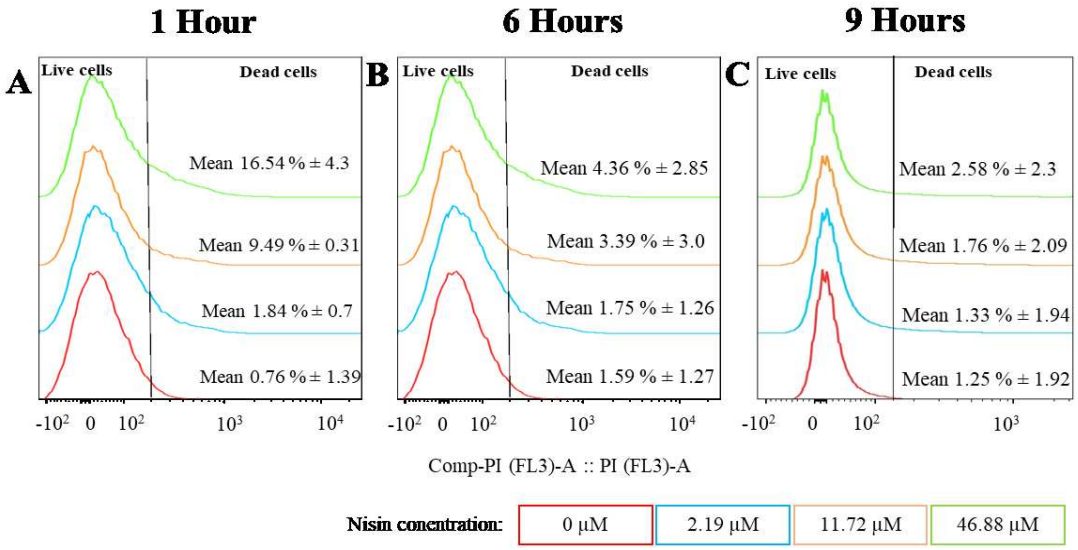

Figure 2: Viability of *S. enterica* under different concentrations of nisin (2.93 µM, 11.72 µM and 46.88 µM) on the flow cytometer after treatment: 1 hour (A), 6 hours (B) and 9 hours (C). The transversal line separates living cells from dead cells. Mean (*n=3*), and standard deviation (±SD) are shown (line). The average of treatments 11.72 µM and 46.88 µM and in A, were significantly different ($p < 0.05$), unidirectional analysis of variance and Tukey Multiple Comparison Test. Means in B and C no different.

It should be considered that the concentration of nisin available in the media is reduced as the number of *S. enterica* cells increased and therefore, the bactericidal effect of this peptide is overcome. Another possibility to explain the decreased of cells death is the adaptation to the cells to the antimicrobial. This adaptation was observed by Prudêncio et al. (2015) when *S. enterica* Typhimurium grew after 24 h in the presence of 115 μM nisin and 1.5 mM EDTA. The author's suggested that nisin resistance in *Salmonella* Typhimurium could be related to changes in the structure of the outer membrane, most likely in the LPS layer. *Salmonella* is recognized by presenting a vast repertory of the adaptive response to stress that can be regulated according to environmental conditions (Hernández et al., 2002). Furthermore, it is common bacterial subpopulations to occur after exposure to the subinhibitory concentration of nisin (Hornbæk et al., 2006) with the Gram-positive bacterium *Listeria monocytogenes*.

3.3 Changes in *S. enterica* biofilm formation in the presence of nisin

Low sub-inhibitory concentrations of nisin did not interfere with biofilm formation by *S. enterica* but, in the concentrations of 46.88 μM, 93.75 μM and 187.5 μM, a significant enhanced of biofilm formation was observed (Figure 3).

Probably, the biofilm formation by *S. enterica* at high concentrations of nisin is a result of the defense response, stimulated by bacteriocin. The biofilm formation is highly regulated, depends on environmental conditions, and confers protection against bactericidal agents (Vogeleer et al., 2014; Yang et al., 2016).

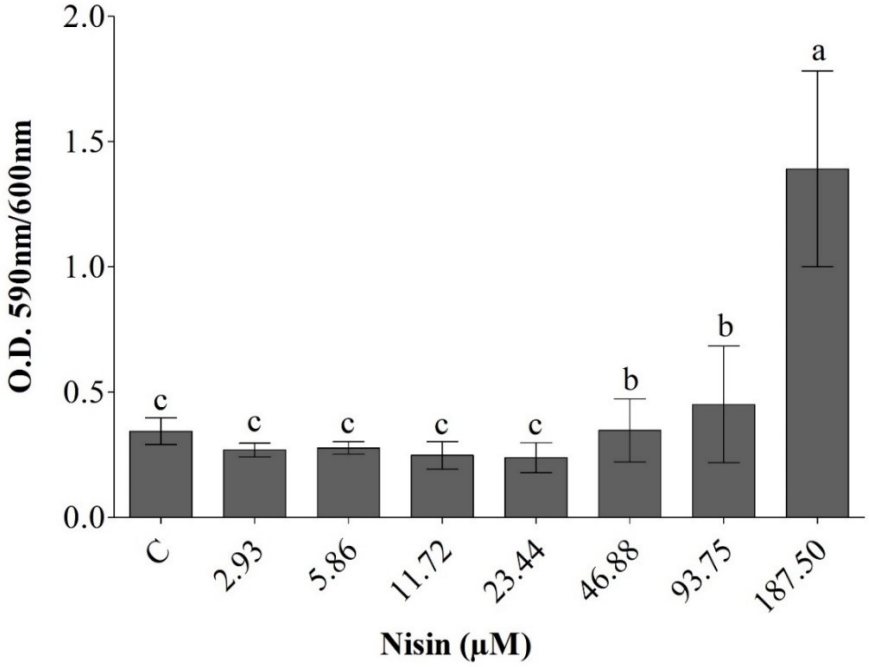

Figure 3: Biofilm of *S. enterica* after 24 hours incubation in BHI media, under different concentrations of nisin (0 - 187.5 µM). Mean (*n=3*), and standard deviation (±SD) are shown (bars). Different letters indicate significant differences based on Tukey's HSD test ($p < 0.05$).

There are some reports on the formation of biofilm by *Salmonella* in the presence of nisin alone or in combination with other agents (Zhao et al., 2016; Pimentel-Filho et al., 2014). High concentrations of nisin of 600, 1,200; 2,400 and 9,600 mg / mL were effective in inhibiting biofilm formation by *Salmonella* Typhimurium, achieve a 90% reduction of biofilm at 24, 48, 72 and 96 hours of incubation, respectively (Yüksel et al., 2018). The combination of P22 phage, EDTA and nisin could also be used to inhibit biofilm formation by *Salmonella* (≥93.2%) at a low phage titer (10^2 Plaque-Forming Units (PFU) / mL), and low EDTA (1.25 mM) and nisin (9.375 mg / mL) concentrations (Yüksel et al., 2018). The effect of nisin on mature biofilms revealed that nisin enhancer its formation, being justified that in a sub-lethal concentration, nisin does not cross the

matrix of exopolysaccharides that surrounds the bacteria, but possibly acts as signaling molecules that induce the formation of biofilm (Yüksel et al., 2018).

Other result focused on the elimination of preformed biofilms by nisin. Mahdavi et al. (2007) observed the elimination of 90% of the biofilm of *Salmonella* Enteritidis in high nisin concentrations (4,000 IU / mL). Reduction of biofilm formed by *Salmonella* Typhimurium was also obtained when nisin was combined with essential oils, such as linalool and p-coumaric acid (Bag and Chattopadhyay, 2017). The increase of nisin activity in biofilm removal was also shown when nisin was combined with antibiotics, demonstrating that the results obtained can be explored for this application. Field et al. (2016) demonstrate that different combinations of concentrations of nisin A and the antibiotics polymyxin and colistin could be adjusted for achieving the best condition for the synergistic activity against the biofilm produced from *P. aeruginosa*.

3.4 Stressor agents increase the susceptibility of *S. enterica* to nisin

Sub-lethal stress conditions under pH in the range of 4.0 to 5.5 and NaCl up to 5% significantly influenced the growth of *S. enterica* (Figure 4), and the stressed cells were more sensitive to inhibition by nisin. These stressors act on the fluidization and destabilization of the outer membrane, permeating the access of nisin to its target site (Prachayasittikul et al., 2007; Back et al., 2014; Matamouros e Miller, 2015; Keerthirathne et al., 2016). The presence of H_2O_2 did not enhance nisin action.

The low pH (4.0, and 4.5) combined with nisin at the sub-MIC concentration increased the susceptibility of *S. enterica* to 5 x after 12 hours of incubation (Figure 4A), even though *Salmonella* spp. presents strategies to resist acid stress. This mechanism is named acid tolerance response (ATR) is activated when the values of pH values are

between 5.5 and 6.0, and, contributes to the survival of the pathogen in food and during the infectious process, protecting *S. enterica* in environments with pH values 3.0-4.0 (Keerthirathne et al., 2016; Ye et al., 2019).

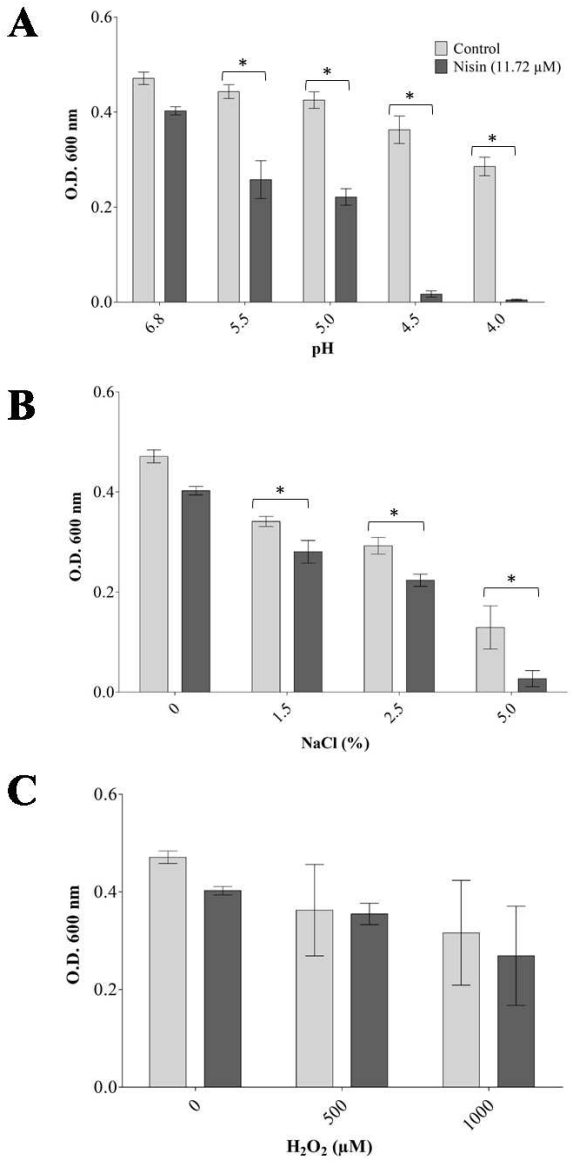

Figure 4: Growth analysis of *S. enterica* after 12 hours incubation under different stress conditions combined with the presence of nisin (11.72 µM). pH (A), concentrations of sodium chloride (B) and hydrogen peroxide (C). Mean (*n=3*), and standard deviation (±SD) are shown (bars). The asterisks indicate significant differences in the same values of pH and NaCl based on the Tukey HSD test ($p < 0.05$).

It has been reported the bactericidal potential of nisin associated with different pH upon this pathogen. *Salmonella* Typhimurium treated with 115 μM nisin showed growth reduction of 1 cycle log_{10} CFU / mL at pH between 5.0 and 6.0 and temperature range of 10 °C to 15 °C (Prudêncio et al., 2016). Reduction in cell growth was especially marked when the 1.5 mM chelating agent (EDTA) was combined, which was effective in reducing of cycles log_{10} (9.0 to 1.0) at high temperatures (35 °C – 45 °C) and near neutral pH (Prudêncio et al., 2016). Khan et al. (2015) optimized the best condition for the antimicrobial activity of nisin (125-150 μg / mL) combined with Na-EDTA (20-30 mM) and pH (5-6), against *Salmonella* Typhimurium. This effect may be justified due to the sensitization of the outer membrane and nisin access to the target site and due to the pH lower, which is ideal for the solubility and antimicrobial activity of nisin. These results reinforce the observations that stressful conditions caused by adjuvants are interesting for the effect of nisin.

The stress promoted by NaCl at 1.5, 2.5 and 5.0 % enhanced the inhibitory effect of nisin, and a reduction of *S. enterica* growth was observed (Figure 4B). Although *S. enterica* was able to grow at concentrations of up to 8% NaCl (Koutsoumanis, 2008; Piu, et al., 2011; Aspridou et al., 2018), this value deviates from the ideal growth limits (Yoon et al., 2013). High NaCl concentration may destabilize external membrane charges and influence the access of nisin (Keerthirathne et al., 2016).

Hydrogen peroxide (H_2O_2) at 1000 μM significantly reduced *S. enterica* growth, however, synergistic effect when combined with nisin at 11.72 μM was not observed (Figure 4C). Considering that hydrogen peroxide forms free radicals of hydroxyl (OH) that attack essential cellular components, including lipids, proteins, and DNA (Back et al., 2014), the reduction of the growth observed can be a result of cell damage caused by the high concentrations of H_2O_2 used (500 μM and 1000 μM).

In general, nisin has been used against Gram-negative bacteria with a co-adjuvant agent to potentiate its effect (Bader et al., 2003; Matamouros and Miller, 2003; 2015; Prudêncio et al., 2016). In this work, it is adopted some combinations of stressing agents, which favored the activity of nisin against *S. enterica*.

3.5 Effect of nisin sub-MIC is accentuated by antibiotics

A significant increase in the inhibition halo of *S. enterica* treated with nisin was observed when in combination with antibiotics, especially with those inhibiting cell wall biosynthesis as ceftriaxone, meropenem, cephalexin and ampicillin; and DNA replication as norfloxacin (Table 2). Increased sensitivity of *S. enterica* to an antibiotic in presence of nisin was also reported by Singh et al. (2013) that showed a decrease in bacterial growth in all combinations of nisin and antibiotics with better results with nisin-ceftriaxone and nisin-cefotaxime both *in vitro* and in *vivo* analyses. The inhibitory effect of nisin was also increased when engineered nisin (S29G) was combined with the polymyxin B nonapeptide against the growth of *E. coli, Salmonella* Typhimurium, and *Cronobacter sakazakii* (Field et al., 2012). Taken together, these results are of great

Table 2: Antibiogram of *S. enterica* using 17 antibiotics combined with nisin in sub-inhibitory concentration of 11.72 µM.

Agent	Code	Concentration (µg)	Inhibition halos (mm)			Mean and standard deviation of the Inhibition halos (mm)		Halo score		Action mode
			R	I	S	Without nisin1	With nisin2	1	2	
Sulfonamides	Sul	300	≤ 12	13-16	≥ 17	18.64 ±1.121	17.22 ± 2.825	S	S	Inhibits nucleic acid replication
Trimethoprim	Tri	5	≤ 10	(11-15)	≥ 16	26.01 ±1.106	27.50 ± 0.020	S	S	Inhibits nucleic acid replication
Levofloxacin	Lvx	5	≤ 13	14-16	≥ 17	26.66 ± 2.130	27.51 ± 0.280	S	S	Inhibits nucleic acid replication
Norfloxacin	Nor	10	≤ 12	13-16	≥ 17	27.60 ± 1.607	28.62 ± 0.923 *	S	S	Inhibits nucleic acid replication
Ceftriaxone	Cro	30	≤ 19	20-22	≥ 23	28.75 ± 1.011	32.13 ± 0.248 ***	S	S	Inhibits cell wall synthesis
Meropenem	Mer	10	≤ 19	20-22	≥ 23	31.62 ± 0.993	33.28 ± 1.673 ***	S	S	Inhibits cell wall synthesis
Cephalexin	Cfe	30	≤ 14	15-17	≥ 18	22.19 ± 0.895	23.95 ± 0.796 *	S	S	Inhibits cell wall synthesis
Ampicillin	Amp	10	≤ 13	14-16	≥ 17	24.42 ± 0.011	27.24 ± 0.023 ***	S	S	Inhibits cell wall synthesis
Cephalothin	Cfl	30	≤ 14	15-17	≥ 18	24.14 ± 1.640	24.46 ± 1.651	S	S	Inhibits cell wall synthesis
Penicillin	Pen	10UI	≤ 28a	-	≥ 29a	12.05 ± 2.482	11.96 ± 0.664	R	R	Inhibits cell wall synthesis
Vancomycin	Van	30	≤ -	-	≥ 17b	0.00	0.00	R	R	Inhibits cell wall synthesis
Oxacillin	Oxa	1	≤ -	-	≥ 18a	0.00	0.00	R	R	Inhibits cell wall synthesis
Tetracycline	Tet	30	≤ 11	(12-14)	≥ 15	23.67 ± 0.971	24.42 ± 0.850	S	S	Inhibits cell wall synthesis
Gentamicin	Gen	10	≤ 12	13-14	≥ 15	19.43 ± 1,406	18.51 ± 3.00	S	S	Inhibits protein synthesis
Erythromycin	Eri	15	≤ 15b	16-20b	≥ 21	0.00	00.00	R	R	Inhibits protein synthesis
Amikacin	Ami	30	≤ 14	15-16	≥ 17	20.62 ± 0.427	21.75 ± 0.4636	S	S	Inhibits protein synthesis
Chloramphenicol	Chlo	30	≤ 12	13-17	≥ 18	26.22 ± 1.273	25.82 ± 1.186	S	S	Inhibits protein synthesis

a: Standard values for *Staphylococcus* spp.

b: Standard values for *Streptococcus* spp.
Asterisks for *P* values: < 0.05 (*), < 0.01 (**) and < 0.001 (***).

importance in the current context of antibiotic resistance and motivate the continued search for alternatives to control microbial growth.

The antibiotics that had the strongest effects in *S. enterica* were those that permeate the physical barriers that the bacteria possess, which prevents the action of nisin. The outer membrane is a barrier to be overcome by several antimicrobials, protecting bacteria from adverse environmental conditions and risk molecules, including many antibiotics and modulates susceptibility to cationic antimicrobial peptides (Ruiz et al., 2006). Considering that bacterial resistance to antibiotics is currently one of the public health problems of global importance, results on the synergistic effect of nisin with antibiotics that destabilize the cell wall is a possible alternative for the control of enteric bacteria, emphasizing the contribution of bacteriocins on medicine area.

3.6 Change the genes expression by nisin at sub-MIC

The concentration of 11.72 μM of nisin did not alter the expression of genes *glgC* and *fliF* related to the biofilm formation in *S. enterica* 1 hour and 6 hours after treatment (Figure 5A). This result corroborates the observation that the biofilm formation was not affected by this sub-inhibitory concentration of nisin (Figure 2). An exception was observed for the gene *fimF*, which was differentially expressed after 1 hour of nisin sub-MIC treatment (Figure 5). In the presence of 46.88 μM nisin, no significant differences in gene expression were observed 1 hour and 6 hours after treatment (Figure 5B). The remaining genes investigated, *glgC*, *fimF*, and *fliF* showed no significant differences in the presence of nisin (11.72 μM and 46.88 μM) 1 hour and 6 hours after treatment (Figure 5).

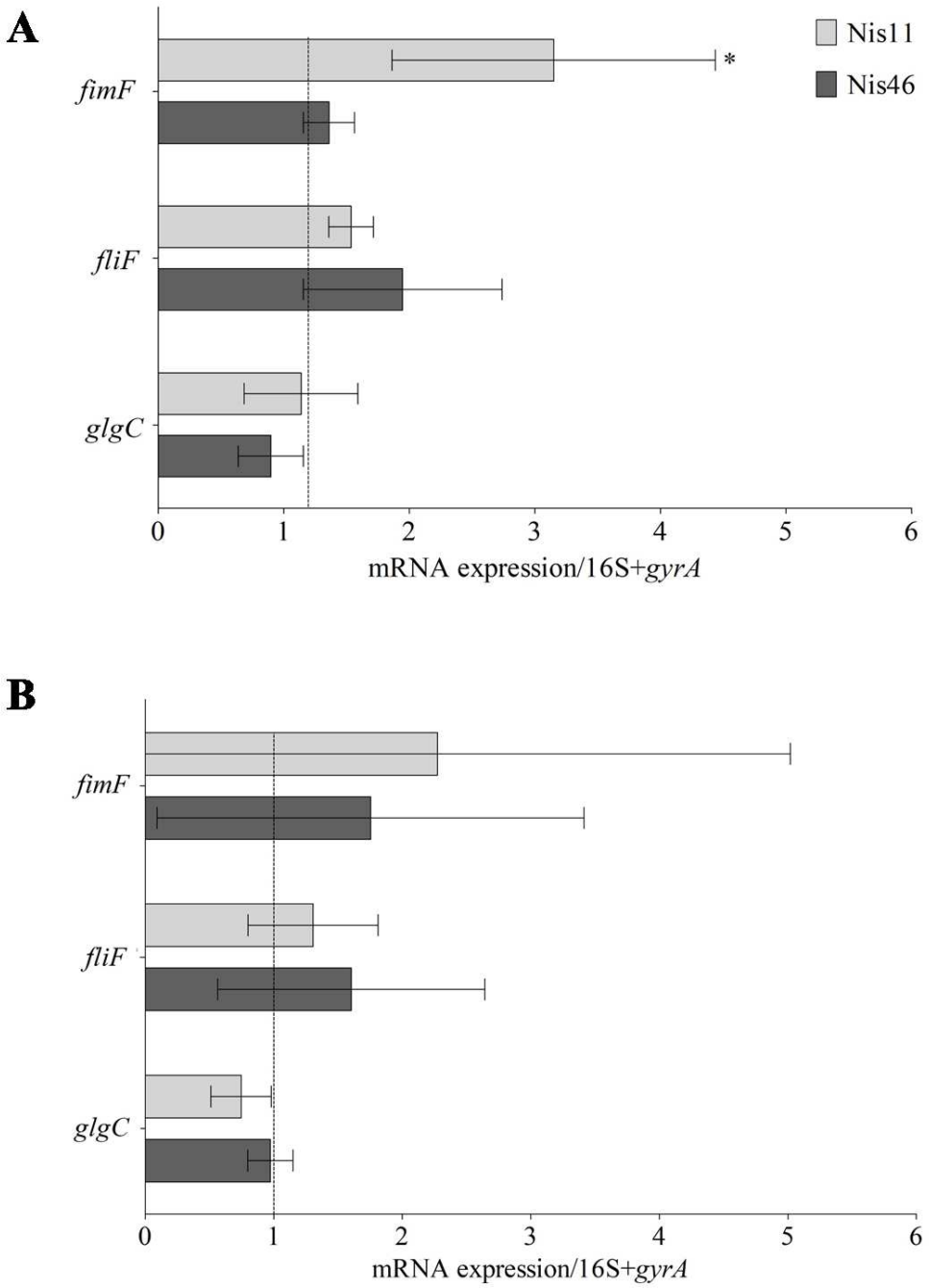

Figure 5: Expression analysis of genes related to biofilm formation of *S. enterica* in the presence of nisin with 1 hour (A) and 6 hours incubation (B). Nis11: inoculated with nisin 11.72 µM; Nis46: inoculated with nisin 46.88 µM. Asterisks indicate significant differences by Tukey's HSD test ($p < 0.001$) compared to those of the control (dashed line). The bars represent the means and vertical bars are standard deviation (SD).

The ability of nisin to regulate gene expression was considered by Pimentel-Filho et al. (2014), and it was observed that the sub-inhibitory concentration of nisin was able to affect the expression of some genes important for biofilm formation in *S. aureus*, such as *icaD* and *fnbA*. Zhao et al. (2016) also determinate that low concentrations of nisin can modulate the expression of more than 600 genes in *S. aureus*, among them, those related to pathogenicity factors, adaptations to atypical conditions, lipid metabolism, RNA synthesis, among others, however, it is not clear how this mechanism occurs.

Others cationic peptides effected the upon gene regulation. Bader et al. (2003) found that polymyxin cationic peptide added with a single fatty acid affected the expression of several genes related to virulence and the stress response of *Salmonella* Typhimurium with the antimicrobial peptides. The genes related to virulence factors of SPI-1 and flagellum synthesis were repressed (Bader et al., 2003). Thus, it is believed that *fimF* superexpression is related to the concentration of nisin and the incubation period adopted in this work.

4 CONCLUSIONS

Membrane destabilizing agents, such as low pH, high concentrations of sodium chloride and antibiotics enhanced the inhibitory activity of nisin on *S. enterica*. In general, it has been observed that although nisin has low Gram-negative growth efficiency, the sub-MIC concentration may change *S. enterica* behavior at the molecular level as genes transcription could be altered such as *fimF*, important for biofilm formation.

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CHAPTER 3

NISIN IN SUBLETAIS DOSES INCREASES THE VIRULENCE OF *Salmonella enterica* IN *Galleria mellonella* LARVAE

ABSTRACT

Salmonella enterica is pathogen inducing self-limiting gastroenteritis and is of worldwide concern. Antimicrobial peptides as nisin emerge as an alternative for the control of microbial growth but its effect on virulence of pathogenic bacteria was not considered. The aim of this work was to evaluate the virulence of *S. enterica* in the presence of sublethal doses nisin using the experimental model *Galleria mellonella*. Nisin sub-MIC at 11.72 μM and 46.88 μM did not affect the cellular viability of *S. enterica* but, promote changes in gene expression within 1 hour of treatment, with significant increases of up to 3-fold of the *pagC* gene. Virulence genes were overexpressed in the presence of subinhibitory concentrations of nisin, as *invA* with 1.8-fold and *invF* with 2.3-fold. Larvae of *G. mellonella* inoculated with 10^3 CFU / *S. enterica* combined or not with 11.72 μM nisin showed similar survival and median lethal time (TL_{50}). However, when *S. enterica* was inoculated in combination with nisin at 46.88 μM, mortality and TL_{50} increased significantly to 50% at 24 hours post-infection (hpi) and 80% at 48 hpi. Infected larvae with *S. enterica* and nisin showed a decrease in overall activity, and the number of bacterial cells recovered from the hemolymph increased after 48 hours of infection. Reduction in hemocytes was more evident at 24 and 48 hours after pathogen inoculation. Defense responses as melanization, nodulation, pseudopodia, immune response and expression of defense proteins of the larvae *G. mellonella* were enhanced when the treatments with *S. enterica* were combined with 11.72 μM and 46.88 μM nisin. These results show an unusual function of nisin, which promotes an increase in virulence of *S. enterica* not yet understood, and that needs to be explored.

1. INTRODUCTION

Salmonella enterica is a Gram-negative pathogenic bacterium with a worldwide highlighting, due to the severe nature of the infection, which can affect several host species, such as chicken, mice, piglets, calves, and humans (Mackenzie et al., 2017). This zoonotic agent can cause food-borne diseases and have a negative impact on public health (Pulido-Landínez, 2019). In human infections, the serovars Typhimurium and Enteritidis are the most reported, inducing self-limiting gastroenteritis called salmonellosis and systemic infection in immunocompromised individuals (Wisner et al., 2012).

Infection in humans occurs through the ingestion of food contaminated with *S. enterica*, which reaches the small intestine, where it finds the ideal conditions to initiate the invasion of epithelial and phagocytic cells (Larock et al., 2015). *S. enterica* survives and replicates in macrophages within vesicles called Vacuole Containing *Salmonella* (SCV), which protects bacteria from the host immune system and offers the ideal conditions for growth (Steele-Mortimer, 2008; Singh et al., 2018).

The success of *S. enterica* infection is related to the presence of virulence genes located in the pathogenicity islands (PI), such as *invA*, *invF* and *hilA*, as well as, virulence plasmids that encode effectors proteins, toxins and components of different types of systems of the secretion, flagella, fimbriae and exopolymers (EPS), important for the invasion, survival, multiplication and escape of the host immune system (Marcus et al., 2000; Wisner et al., 2012). In addition, *S. enterica* can resist to acid stress and the presence of cationic antimicrobial peptides (CAMPs) within macrophages through the action of the two-component PhoPQ system and PagC and PagP proteins (Nishio et al., 2005; Matamouros et al., 2015; Hwang-Soo et al., 2016).

Nisin is a CAMP that interacts with lipid II present in the bacterial membrane, inhibiting cell wall synthesis and forming pores in the inner membrane (Hasper et al., 2006; Perez et al., 2015). Unlike Gram-positive bacteria, where nisin has easy access to lipid II, in Gram-negative, this mechanism is prevented by the outer membrane, which confers protection and making it anionic repulsing the nisin binding at the target site (Matamouros and Miller, 2015). Despite this, various studies have shown the application of nisin combined with factors that favor the permeation of the outer membrane and potentiate its effect against Gram-negative bacteria, including those with antibiotic resistance (Singh et al., 2013, 2014; Prudêncio et al., 2015a, 2016; Field et al., 2016).

Galleria mellonella has been widely used in recent years as an experimental model for microbial virulence assay (Fedhila et al., 2010; Ramarao et al., 2012; Tsai et al., 2016; Blanco et al., 2017; Barnoy et al., 2017; Parthuisot et al., 2018), since it presents advantages of application, as the innate immune response similar to mammals, easy manipulation, besides reducing the use of the murine model in the preliminary phase of the investigation (Trentin, 2016). The response to infectious agents of *G. mellonella* occurs at the cellular and humoral level (Morelo and Trentin, 2016; Wojda, 2017; Pereira et al., 2018). The cellular response is mediated by hemocytes, which are different types of cells found in the hemolymph, which act on phagocytosis, encapsulation, coagulation, and nodulation, while the humoral response is composed of soluble molecules that immobilize or eliminate the pathogen, such as complement-type proteins (opsonins), melanin and antimicrobial peptides (AMPs) (Morelo and Trentin, 2016; Wojda, 2017; Pereira et al., 2018).

In the present study, the virulence and stress resistance of *S. enterica* in the presence of sublethal doses nisin during infection in *G. mellonella* was investigated.

2. MATERIALS AND METHODS

2.1 Bacterial strain

Salmonella enterica subspecies *enterica* serovar Enteritidis phage type 4 (PT4) 578, isolated from chicken meat, provided by Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Brazil) was used in this study and has been previously described (Campos-Galvão et al., 2015). The stocks culture was performed in brain and heart infusion broth (BHI, Oxoid, England), supplemented with 20% (v/v) sterilized glycerol and stored at -20 °C. Before the experiments, the bacterial cultures were reactivated in BHI for 24 hours at 37 ± 1 °C aerobically. After growth, cells were centrifuged at 6.000 *g* for 10 minutes, washed twice in 0.85% saline. The inoculum was standardized to 0.1 of optical density at 600 nm (OD_{600nm}) (approximately 10^7 CFU / mL) using a spectrophotometer (Thermo Fisher Scientific, Finland).

2.2 Preparation of the nisin solution

The stock solution of nisin was prepared by dilution of 1.000 g of Nisaplin (2.5% Nisin, Nisaplin®, kindly provided by Danisco Brazil) in 5 mL of sodium phosphate solution (5 mM phosphate buffered saline (PBS), pH 2.0). The final stock concentration was 1,500 μM. After preparation, the stock solution was stored under refrigeration until the beginning of the experiments.

2.3 Analysis of cell viability in the presence of nisin

The viability of *S. enterica* PT4 was analyzed on the flow cytometer in the presence of in sub-inhibitory concentrations sub-MIC of 11.72 μM, equivalent to 1,572.04 μg of nisin, and 46.88 μM, equivalent to 6,288.17 μg nisin, defined in preliminary studies. Cells in the exponential phase were centrifuged at 6.000 *g* for 10 minutes and washed twice with 0.85% saline and standardized to a concentration of approximately 10^6 CFU / mL. Propidium iodide (PI) was then, added for analysis of cell viability. Readings of viability were performed using a BD FACSVerse cytometer (BD Bioscience, California, USA) at Núcleo de Microscopia e Microanálise da UFV NMM / UFV. Cells without nisin were used as controls.

2.4 Analysis of the gene expression of *S. enterica*

The *invA*, *invF*, *hilA*, and *pagC* genes were amplified by qRT-PCR to evaluate the virulence response and resistance of *S. enterica* in the presence of nisin sub-MIC. Therefore, previously activated *S. enterica* cultures (10 mL) were incubated with 11.72 μM or 46.88 μM of nisin at 37 °C for 1 hour and 6 hours. Cells without nisin and those treated with PBS pH 2.0 were used as negative controls. After, the samples were centrifuged to obtain the total biomass and subsequent extraction of total RNA. Cells were homogenized with 600 μL of TRI Reagent® (Sigma-Aldrich, Canada) for 30 seconds in a vortex and incubated for 15 minutes at room temperature. Then, 200 μL of ice-cold chloroform followed by manual homogenization for 15 seconds, incubation for 2 minutes at room temperature and subsequent centrifugation at 12,000 *g*, 4 °C for 15 minutes for separation of aqueous and organic phases. The aqueous phase was transferred to a new 1.5 mL microtube, added with 500 μL of isopropanol, incubated for 15 minutes at room temperature to precipitate the RNA and

centrifuged at 12,000 *g*, at 4 °C for 10 minutes. The supernatants were discarded, and 1 mL of ice-cold ethanol (75%) was added to the microtubes, homogenized and centrifuged at 7,500 *g*, 4 °C for 10 minutes. The supernatants were discarded, and the precipitated total RNA was allowed to air dry for the addition of RNase-free water for solubilization of the extracted RNA. The concentrations of the total RNA were evaluated by spectrophotometer reading at 260 nm and 280 nm to calculate the 260/280 ratio. The quality of the RNA was evaluated by electrophoresis in 1.5% (w/v) agarose gel. Total RNA was purified to eliminate the DNA with DNAse I (Promega, Madison, USA). From 1 µg of RNA, the syntheses of the first cDNA strands were performed according to the protocol recommended by the manufacturer of the ImProm- II^{TM} reverse transcription system (Promega, Madison, USA). The RT-qPCR was performed in a reaction with a volume of 12.0 µL containing 1.0 µL of the cDNA template, 1.0 µL of the respective primers (Table 1), 6.0 µL SYBR Green master mix and 3.0 µL of sterile ultrapure water. Reactions were performed with the first step at 50 °C for 3 minutes, the second step of 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. All amplifications were performed on optical grids in 96-well plates in the Bio-Rad C1000 Manager sequence detector system. The fluorescence data were processed using the Bio-Rad CFX Manager software, resulting in cycle limit values for each sample. This experiment was performed with three independent replicates, and each sample was evaluated in triplicate. The gene dosages were obtained by the $2^{-\Delta\Delta Ct}$ method, and the subunit of the DNA gyrase and 16S ribosomal was used as an endogenous control to correct the dosages of the genes of interest, and the control treatment was used as a target.

Table 1. Primers used in this study

Primer	Sequences (5'–3')	Reference
*invA*F	CGTTTCCTGCGGTACTGTTAATT	Li and Chen, 2013
*invA*R	TCGCCAATAACGAATTGCCCGAAC	Li and Chen, 2013
*invF*F	ATGATTAACGGCTAATTGGGTGA	Campos-Galvão et al., 2015
*invF*R	CGGAAAAGCGAAGAGTGAATTAC	Campos-Galvão et al., 2015
*hilA*F	GCTGCACCAGGAAAGCATTAA	Campos-Galvão et al., 2015
*hilA*R	GCGAAGTCCGGGAATACATC	Campos-Galvão et al., 2015
*pagC*F	GGGTCTGTTGAGCCTGAAGG	Bader et al., 2003
*pagC*R	GCCATCCTGAGTGGAATGTTC	Bader et al., 2003
*gyrA*F	CCAATACGTTCATGGCGTAAAG	Campos-Galvão et al., 2015
*gyrA*R	GATTATGCGATGTCGGTCATTGT	Campos-Galvão et al., 2015
*16SrRNA*F	CGTGTTGTGAAATGTTGGGTTAA	Kollanoor Johny et al., 2017
*16SrRNA*R	CCGCTGGCAACAAAGGATAA	Kollanoor Johny et al., 2017

2.5 Investigation of virulence of *S. enterica* in *G. mellonella*

The larvae of *G. mellonella* used in this work were donated by the Laboratory of Molecular Genetics of Microorganisms at the Universidade Federal de Viçosa (UFV) and were selected in a standardized manner, observing morphological characteristics, like weight, color, and fast movement. The larvae were inoculated directly into the hemolymph using 0.3 mL of insulin syringes (Becton Dickinson - BD, United States), and after were incubated at 37 °C and individually monitored for pigmentation (melanization) production and time of death for 96 hours. Larvae without movement in response to touch were considered dead (Pereira et al., 2015; Blanco et al., 2017).

Virulence bioassay

In the virulence bioassay the bacterial suspension corresponding to, approximately, 10^3 CFU per larva, diluted in PBS with pH 6.8, was mixed with nisin at concentrations of 11.72 μM and 46.88 μM and immediately injected into the hemolymph. Then, the larvae were incubated and examined, as previously mentioned. All tests involving the inoculation of bacteria in *G. mellonella* larvae were performed in two experimental triplicates (n = 60 larvae per treatment). Larvae injected with PBS, nisin (Nis 46.88 μM) and not inoculated (Naive) were used as negative controls (Ramarao et al., 2012; Pereira et al., 2015).

Walking behavior

For this bioassay, a computerized video tracking system (ViewPoint LifeSciences) was used to record larvae walking in an arena consisting of an open Petri dish 9 cm, in diameter and 2 cm high, for 10 minutes. This method allowed the evaluation of possible sub-lethal effects in locomotion, walking speed (cm s^{-1}), distance (cm), resting time (s) and the number of stops for larvae after 24 hours incubation (Tomé et al., 2012). Five larvae were sampled in four repeats totaling 16 larvae per treatment (Bernardes et al., 2018).

Hemolymph collection

The hemolymph was collected from five infected larvae within each treatment and pooled to complete 100 μL (Harding et al., 2012) at 0, 4, 24, and 48 hours after the injection. The samples were transferred to 1.5 mL microtubes containing cold anticoagulant solution with pH 4.5 (Mead et al., 1986) for further analysis.

Bacterial growth monitoring

To monitoring bacterial growth, the hemolymph samples were collected and serially diluted with saline (0.85%), followed by plating on BHI agar and XLD (deoxycholate-lysine-xylose agar, Becton Dickinson - BD, USA). The plates were incubated at 37 °C for 24 hours for colonies counting (Pereira et al., 2015). The experiments were performed on three replicates and the results were expressed as CFU per larva.

PCR of colony

A small portion of single colony of *S. enterica* was removed on the surface of an XLD Agar with an autoclaved toothpick and mixed with GoTaq® Green Master Mix (M712) (containing Taq DNA polymerase, dNTPs, $MgCl_2$ and blue and yellow dyes) (Promega Corp., USA) together with the oligonucleotides pair DNA correspondent to gene *invA* (Table 1) according to the manufacturer's instructions. The parameters of the PCR were conducted by incubation at 94 °C for 10 minutes and then cyclized 30 times during the steps: denaturation (94, 30 seconds), annealing (55, 40 seconds), primer extension (72, 40 seconds). The final extension was at 72 °C for 5 minutes and the amplified fragments were separated by 1.2% agarose gel electrophoresis according to the modified protocol of Sunar et al. (2014).

Quantification of hemocytes

The hemolymph collected as described above were diluted in cold anticoagulant and used for total hemocytes counts using a standard Neubauer chamber of 10 µL capacity (Kasvi, Brazil). The 2-squares cells were counted using a light microscope (Olympus CX41, 40 x objective) in three independent replicates according to the modified protocol of Blanco

et al. (2015). The total cell number was calculated according to the formula described below. The results were expressed as hemocytes per µL.

$$Nº\ hemocytes/\ mL = \frac{Nº\ hemocytes\ counted}{Nº\ of\ quadrants\ counted}\ x\ dilution\ factor\ x\ 10^4$$

Histology

The hemolymph samples from each treatment were transferred to 1.5 mL microtubes containing the Zamboni fixative (2% paraformaldehyde containing 15% optical acid in 0.1 M sodium phosphate buffer) and incubated at room temperature for 15 minutes. Then the samples were centrifuged at 200 *g* for 10 minutes and washed three times with PBS (0.1 M, pH 7.2) for further histopathological and immunofluorescence analysis (Fernandes et al., 2019).

The histopathological analyses were according to Pereira et al. (2015), with modifications. The hemocytes samples were centrifuged at 200 *g* for 10 minutes, the pellet was maintained in a microtube, dehydrated in a growing series of ethanol (70-100%) and embedded in historesin (Leica, Wetzlar, Germany). The samples were incubated in vacuum desiccator and then sectioned (1 µm) in the microtome (Leica RM2255, Germany) to obtain sections subsequently stained with toluidine blue for observation under an optical microscope (Olympus, Japan).

Cytoskeleton

After fixation, the hemolymph was centrifuged at 200 *g* for 10 minutes and washed with PBS (0.1 M, pH 7.2). Then the hemocytes cytoskeleton was stained with Rhodamine Phalloidin (filamentous actin; Sigma-Aldrich, Canada) and nuclei with DAPI (4′-6-diamidino-2 phenylindole; Biotium, Hayward, California, USA) as previously described (Hwang et al., 2015; Kwon et al., 2014). The samples were mounted glass slides with Mowiol anti-fading medium (Fluka, Switzerland), and analyzed by microscopy (Olympus BX53 coupled to the Olympus DP73 digital camera (Olympus Corp., Tokyo, Japan)) from the Laboratório de Sistemática Molecular, Departamento de Biologia Geral, Universidade Federal de Viçosa (DBA/UFV).

Immunofluorescence

The samples of hemolymph fixed described above were centrifuged at 200 *g* for 10 minutes and washed for 15 minutes with 1% PBS-Triton-X100 (PBS-T) and then, incubated at 4 ºC with the primary antibodies, anti-peroxidase (1: 500) (Cell Signaling Technology, Inc., Beverly, MA, USA) and anti-cactus 3H12 (1: 500) (Developmental Studies Hybridoma (DSHB), Iowa City, Iowa, USA). After 24 hours incubation, samples were centrifuged at 200 *g* for 10 minutes and washed with PBS, then incubated with secondary antibody anti-rabbit IgG-FITC (Sigma-Aldrich; 1:500) in PBS for 24 hours at 4 °C. Then, samples were centrifuged at 200 *g* for 10 minutes and washed with PBS and then stained for 30 minutes with 4′,6-diamidino-2-phenylindole (DAPI) for DNA staining. The samples were mounted glass slides with Mowiol anti-fading medium (Fluka, Switzerland). Stained cells were observed under a fluorescence microscope (Olympus BX53, Japan), equipped with a monochrome and color camera, with filters for DAPI, FITC, tetramethylrhodamine

isothiocyanate, and Cy5 and an XM10 monochrome camera for microscopic fluorescence images (1376 × 1032-pixel resolution) from DBA/UFV. For flow cytometry, hemocytes were quantified by using a cytometer (BD Bioscience, California, USA) at NMM / UFV. For an immunostaining negative control, cells were treated as described above, except for incubation with the primary antibody (Blanco et al., 2015).

Melanization assay

Melanization was quantified in hemolymph samples (n=12 for each treatment) collected in the previous step. The samples were mixed in anticoagulant, pH 4.5 1:1 and were transferred to 96-well plates. After 5 minutes, the optical densities of the wells were read in a spectrophotometer (Thermo Scientific Multiskan GO, USA) with a wavelength of 405 nm (Jorjão et al., 2018).

Infection of G. mellonella by S. enterica expressing green fluorescent protein (GFP)

The suspension corresponding to, approximately, 10^5 CFU of *S. enterica* per larva content plasmid green fluorescent protein (GFP) expression, resistant Kanamycin (Plasmid BBa_I120260, iGEM, Bostom, Massachusetts, USA), was injected into the hemolymph of *G. mellonella* as previously mentioned and posterity incubated for one hour for examination under fluorescence microscope (Olympus Corp., Tokyo, Japan).

2.6 Statistical analysis

Kaplan-Meier was used to estimate survival curves and the median Lethal Times (LT_{50}). Curves similarity was tested using χ^2 Log-Rank test and paired comparisons were carried out using the Bonferroni correction ($p < 0.05$). A dose-response model (Generalized

Linear Model, link= *probit*) was also fitted to estimate the Lethal Dose (LD_{50}) of *S. enterica* inoculated. The analysis bacterial, and bioassay with larvae were performed by one-way analysis of variance (ANOVA) and Tukey's HSD test for multiple comparisons across the levels of the explanatory variable ($p < 0.05$). Residuals were checked to verify the adequacy of the distribution in all models. The analyses were performed using R software (version 3.4.4; R Core Team, 2018).

3 RESULTS AND DISCUSSION

3.1 Nisin does not interfere in the viability of *S. enterica*

The cell viability of *S. enterica* was maintained shortly after treatment with nisin at concentrations of 11.72 μM and 46.88 μM (Figure 1). The maximum proportion of dead cells was, approximately, 7% and this indicates that nisin does not immediately interfere with the viability of *S. enterica* ($F_{2, 6} = 3.23$, $p = 0.11$). Similarly, the results obtained by Galvão et al. (2015) showed that the viability of nisin-treated *Salmonella* Typhimurium (500 AU / mL) did not differ statistically during the first hour. The low efficacy of nisin against *S. enterica* occurs due to the presence of the outer membrane that the Gram-negative bacteria, acting as a physical barrier and protecting the cell against antimicrobials (Prudêncio et al., 2015b; Li et al., 2018). However, when nisin is combined with treatments as chelating agents, plant essential oils, acidic conditions, heating, freezing or high-pressure processing, its effect upon Gram-negative bacteria is enhanced (Singh et al., 2013, 2014; Prudêncio et al., 2015b; Li et al., 2005; 2018).

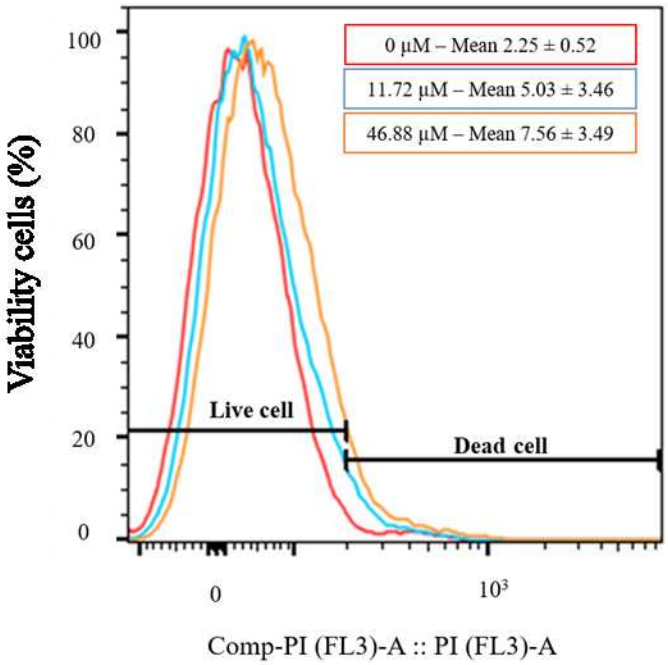

Figure 1: Viability of *S. enterica* on the flow cytometer under sub-MIC concentration of 11.72 µM and 46.88 µM of nisin of meditated after one hour of treatment. Lines separate living cells from dead cells. Mean (*n=3*), and Standard Deviation (± SD) are shown (line). The Means no different ($p = 0.11$).

3.2 Nisin induces overexpression of virulence and resistance to stress genes in *S. enterica*

Although little variations in live cells of *Salmonella* were observed in the presence of sub-MIC concentrations of nisin, the expression of the virulence and resistance genes was altered in relation to the control after 1 hour of incubation. The *pagC* gene, related to resistance, increases significantly up to 3-fold when *S. enterica* was exposed to nisin at sub-MIC concentration of 11.72 µM ($F_{4, 20} = 46.21$, $p < 0.001$; Figure 2A), while virulence genes were overexpressed at both concentrations of nisin, being *invA* with 1.6-fold and 1.8-fold and *invF* with 2.3-fold and 2.2-fold in 11.72 µM and 46.88 µM, respectively. In contrast, the

expression of the *hilA* gene remained unchanged compared to that of the control in analyzed conditions ($F_{12, 52} = 29.79$, $p < 0.001$; Figure 2A; B).

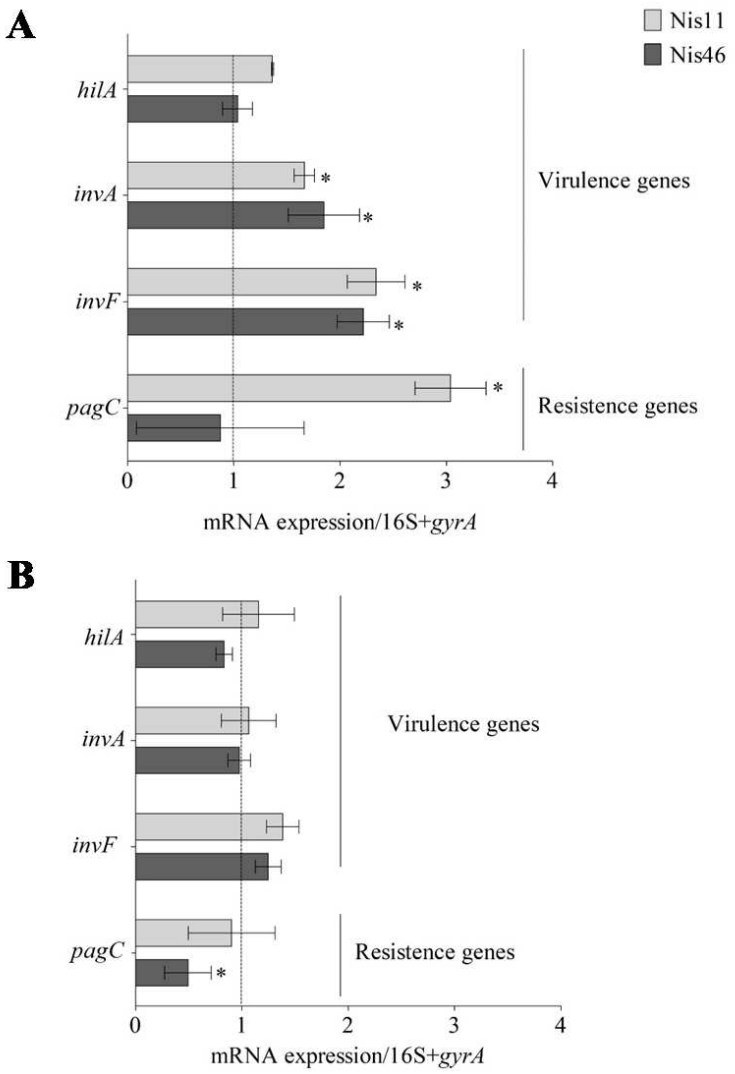

Figure 2: Transcription of different classes of genes (resistance and virulence) is altered after the growth of *S. enterica* in the presence of nisin with 1 hour (A) of incubation and 6 hours (B). The most prominent genes that were differentially regulated by growth with nisin (Nis11: inoculated with nisin 11.72 μM; Nis46: inoculated with nisin 46.88 μM). Asterisks indicate significant differences by Tukey's HSD test ($p < 0.001$) compared to those of the control (dashed line). The bars represent the means and vertical bars are standard deviation (SD).

The increase in expression of the virulence genes, *invA* and *invF* when *Salmonella* was in the presence of 11.72 μM nisin suggests a response to the stress condition caused by nisin. This increase in the expression of virulence genes already been registered in the presence of other stressors such as low values of pH and oxygen tension, osmolarity, bile salts, Mg2 + ions, short-chain fatty acid concentration (SCFAS) (Clements et al., 2001; Lim et al., 2007; Martínez-Flores et al., 2016). The induction of *invA* and *invF* genes in *S. enterica in vitro* occurs during the initial stationary phase and their expression is regulated by the growth phase, and other environmental conditions (Lim et al., 2007; Martínez-Flores et al., 2016). So, nisin can act as one of these stress conditions and induce the expression of these virulence and resistance genes. Positive regulation in genes of several pathogenicity factors was observed in *S. aureus* exposed to nisin (Zhao et al., 2016), reinforcing hypothesis that nisin is able to modulate virulence and resistance phenotype expression, acting as a signal of gene expression and altering the transcribed profile (Zhao et al., 2016; Chikindas et al., 2018). The infection process of the entomopathogenic bacterium *Photorhabdus luminescens* and virulence and resistance phenotype is associated with the positive regulation of resistance genes, virulence and bacterial antagonism related to resistance to CAMPs (Mouammine et al., 2017).

The increase *pagC* expression observed in *S. enterica* in the presence of 11.72 μM of nisin indicates that this bacteriocin may be causing some type of stress in cells, inducing a resistance response whereas, PagC is an outer membrane protein, essential for virulence and survival within macrophages, and its regulation is mediated by the two-component systems PhoPQ, responsible for AMP resistance (Nishio et al., 2005; Matamouros et al., 2015; Hwang-Soo et al., 2016). A similar situation was observed when *S. enterica*

Typhimurium was exposed to cationic peptides polymyxin, α-helical C18G, and protegrin-1, and there was an increase in the expression of *pagC* up to 17-fold in the presence of polymyxin (Bader et al., 2003).

The involvement of microbial peptides, including nisin, in the regulation of several processes in a microbial community, was recently considered. Besides their primary function as an antimicrobial, bacteriocins can signalize some mechanisms (Chikindas et al., 2018). In summary, our results indicate that nisin is capable of inducing a positive response in resistance and virulence genes in *S. enterica* cells and that this response did not correlate with the nisin concentration at times of 1 and 6 hours after incubation, since this increase in gene expression was not evidenced in the concentration of 46.88 μM of nisin.

3.3 *S. enterica* reduces the survival of *G. mellonella*

An analysis of the virulence of *S. enterica* in the *G. mellonella* model was performed, and data obtained with the survival curve indicated that the larvae survival rate during the analyzed period was dose-dependent (Log-Rank: $\chi^2 = 305$, df = 4, $p < 0.001$) (Figure 3). Intrahaemocoelic injection of PBS and *S. enterica* at the dose of 10^2 CFU per larva had no measurable negative impacts on larval survival, and no mortality occurs in control, PBS, and naive larvae (Figure 3A). The higher larvae mortality rate was observed when 10^4 CFU was injected, and the mortality rate gradually decreased as the dose of bacteria was reduced (Figure 3A). The bacterial inoculation of 10^3 CFU per larvae showed more significant LT_{50} than the 10^4 CFU dose (Figure 3B) and was closer to the lethal dose (DL_{50}) (± standard error) estimated (696.52 CFU of *S. enterica* suspension / *G. mellonella*) (±

291.91). Thus, the bacterial inoculum of approximately 10^3 CFU was adopted for the following bioassays to evaluate the virulence of nisin-treated *S. enterica*.

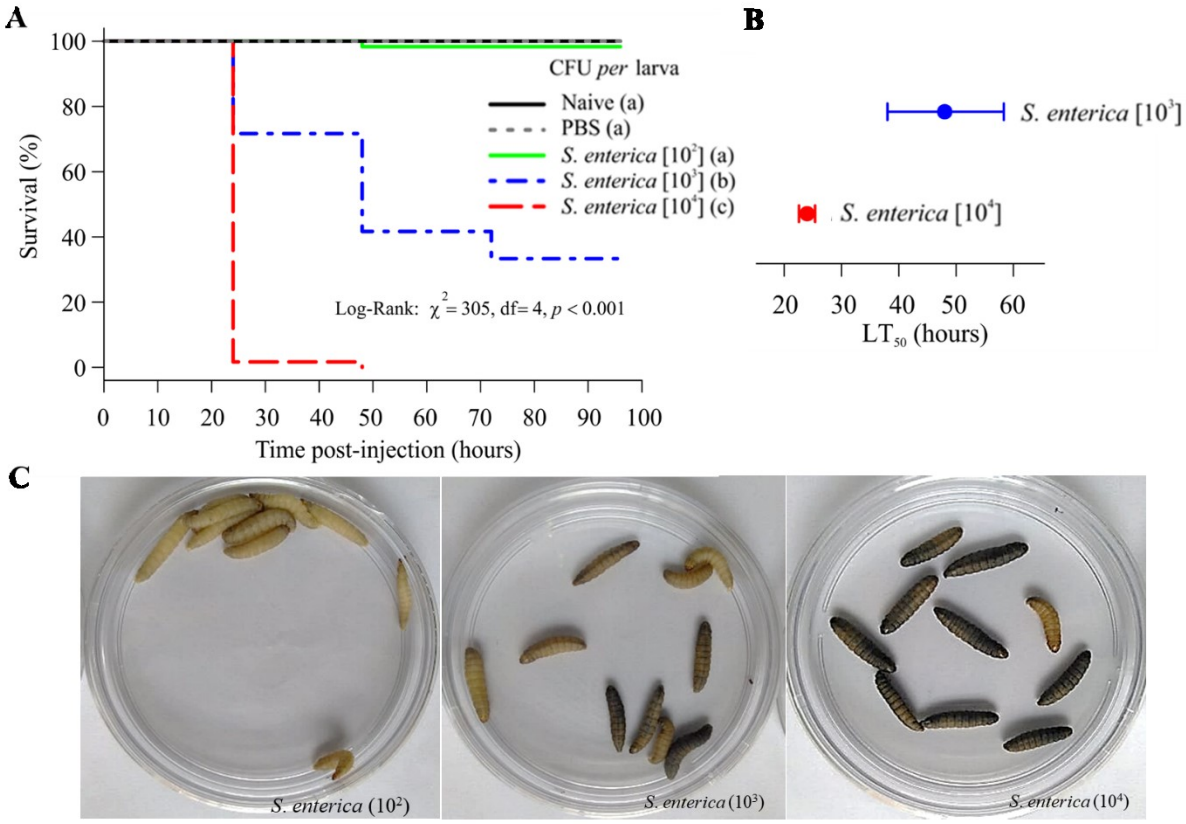

Figure 3: Survival curves (A) and LT_{50} (median ± confidence limits) (B) of *G. mellonella* inoculated with *S. enterica* at different concentrations of bacteria, 24 hours post-infection. Different lower-case letters indicated significant differences by the Bonferroni method ($p < 0.05$). Larvae with melanization enhanced by infection bacterial 24 hours post-infection (C). Naive: not inoculated; PBS: inoculated with PBS pH 7.0; *S. enterica*: inoculated with *S. enterica* approximately 10^2, 10^3, and 10^4 CFU *per* larva.

Larvae of *G. mellonella* have been adopted experimentally in other works for research with pathogenic bacteria as well as *S. enterica*. Bender et al. (2013), after bioassays using a variety of inoculum doses, established that the LD_{50} of *Salmonella* Typhimurium was

3.6 x 10^3 bacteria per larva. Viegas et al. (2013) confirmed the use of a *G. mellonella* model in *S.* Typhimurium in pathogenicity studies through the parallel analysis of a mutant with a mutation in *hfq*, obtained similar to the ones reported in a mouse model. Analysis of the virulence profile for pathotyping using the *G. mellonella* was used for 13 isolates *S. enterica*, (chicken invasive *Salmonella* serovar Enteritidis or *Salmonella* serovar Gallinarum, a multidrug-resistant, *S*. Kentucky, and a *S*. Typhimurium DT104) resulted in high mortality of the larvae (Card et al., 2016).

The larvae had different phenotypes according to the aggressiveness of the treatments used, such as black pigmentation (Figure 3C), mobile reduction and cocoon production, which intensified in the treatments combined with nisin (data not shown).

The dose-dependent response to pathogens by *G. mellonella* was observed by Pereira et al. (2015) when analyzing the virulence of 21 clinical isolates of *Actinobacillus pleuropneumoniae* and these authors concluded that larval mortality was also directly related to the virulence of the strains used, and the incubation temperature adopted after inoculation. Parthuisot et al. (2018) also demonstrated that the inoculation of *Xenorhabdus nematophila* 10 and 100-fold dilutions promoted a quick death of *G. mellonella* after injection, differently from the 1000-fold dilution, in which the larvae were less affected and were used for further analysis.

3.4 The virulence of *S. enterica* is potentiated by nisin

Nisin at a concentration of 46.88 μM did not interfere with larval survival, and similar results were observed with the controls treatments with, PBS or naive (Figure 4A).

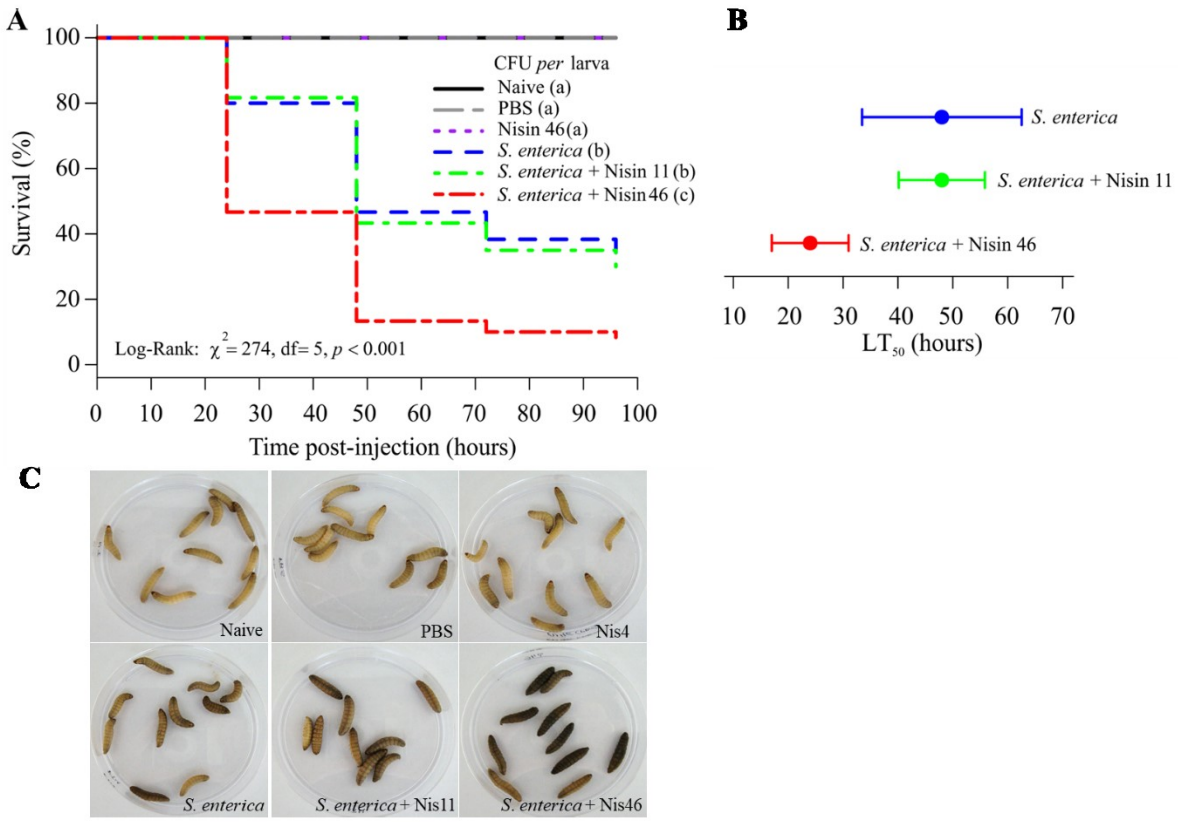

Figure 4: Modulation of the virulence of *S. enterica* by nisin. Survival curves of *G. mellonella* inoculated with *S. enterica* with different concentrations of nisin (A). LT_{50} (median ± confidence limits) (B). Different lower-case letters indicated significant differences by the Bonferroni method ($p < 0.05$). Larvae with melanization enhanced by bacterial 24 hours post-infection (C). Naive: not inoculated; PBS: inoculated with PBS pH 7.0; Nis46: inoculated with nisin 46.88 µM; *S. enterica*: inoculated with *S. enterica* (10^3); Nis11 and Nis46: inoculated with *S. enterica* (10^3) with nisin 11.72 µM and 46.88 µM, respectively.

However, inoculation of *S. enterica* combined with nisin at 11.72 µM reduced the survival of *G. mellonella* larvae, when *S. enterica* was inoculated with 46.88 µM nisin compared with control (Log-Rank: $\chi^2 = 274$, df = 5, $p < 0.001$; Figure 4A). Larvae inoculated with 10^3 CFU of *S. enterica* alone or combined with 11.72 µM nisin presented similar survival and LT_{50}

(Figure 4B). However, when *S. enterica* was inoculated in combination with nisin at 46.88 μM, the survival and LT_{50} significantly decreased (Figure 4). The progression of *S. enterica* infection resulted in increased black pigmentation of *G. mellonella* throughout the 24 hours post-incubation (Figure 4C). These results show an unusual behavior of nisin, which promotes an increase in the virulence of *S. enterica*.

The reduces in the survival rate of *S. enterica*-infected larvae combined with nisin may be related to the ability of nisin to induce the overexpression of virulence and resistance genes previously observed (Figure 2A). It has been demonstrated that the phenotype of CAMP resistance in the entomopathogenic bacterium *P. luminescens* increased the capacity of infection and survival in insects and corpses (Mouammine et al., 2017).

3.5 The activity of *G. mellonella* is reduced in the presence *S. enterica* exposed to nisin

Through video tracking, it was possible to monitor the larvae movement and obtain standardized information about the phenotypic changes resulting from the infectious process. It was observed that the activity of the larvae was significantly different between treatments ($F_{5, 24} = 23.1$, $p < 0.001$, Figure 5A). The general pattern of larval activity showed sub-lethal effects on the locomotion of control larvae inoculated with PBS, nisin and, those inoculated with 10^3 CFU of *Salmonella* (Figure 5). Significant reduction in group activity treated with *Salmonella* and nisin varied in a progressive manner (Figure 5A). These results lead us to conclude that nisin promotes an increase in the virulence of *S. enterica* that are interfered with the behavior of *G. mellonella* larvae (Figure 5B).

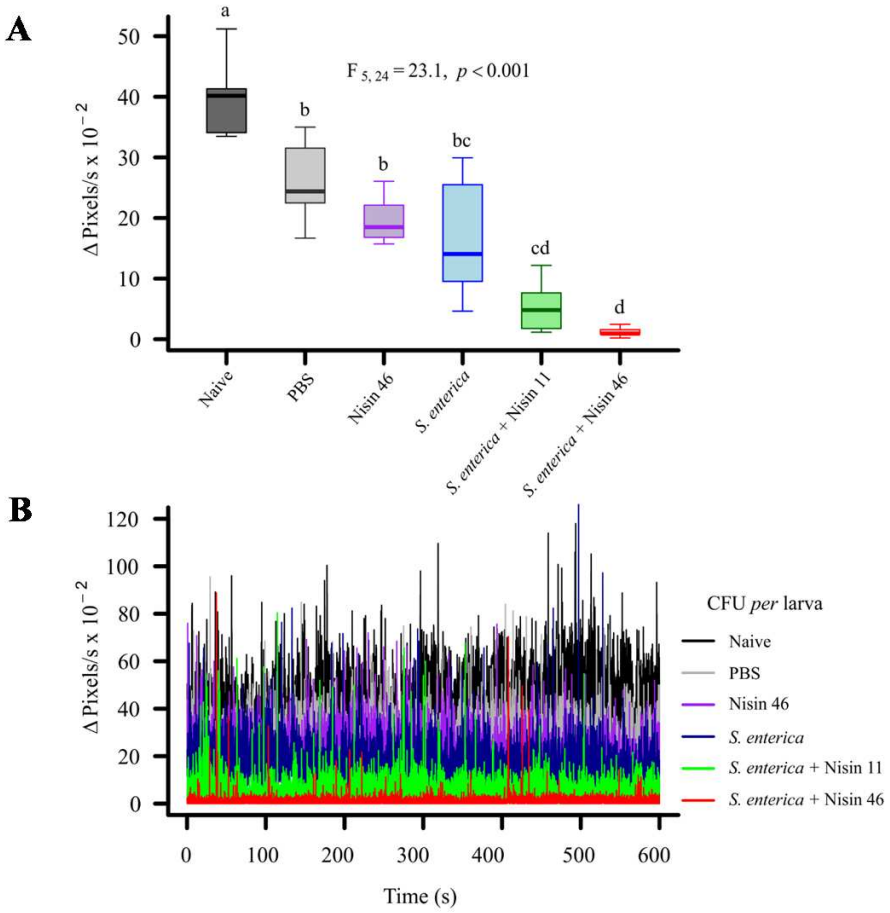

Figure 5: Activity of *Galleria mellonella* after 24 hours of inoculation of *S. enterica* at different concentrations of nisin. Box plot indicates the median (solid line), and dispersal (lower and upper quartiles) of the overall activity (A). Different letters indicate significant differences by Tukey's HSD test ($p < 0.05$). Activity profile recorded for 10 minutes (B).

3.6 Nisin increased multiplication of *S. enterica* in the hemolymph of *G. mellonella*

The quantification of the *S. enterica* cells recovered from the hemolymph showed the permanence of *S. enterica* during the 48 hours of monitoring, except in the controls (Figure 6).

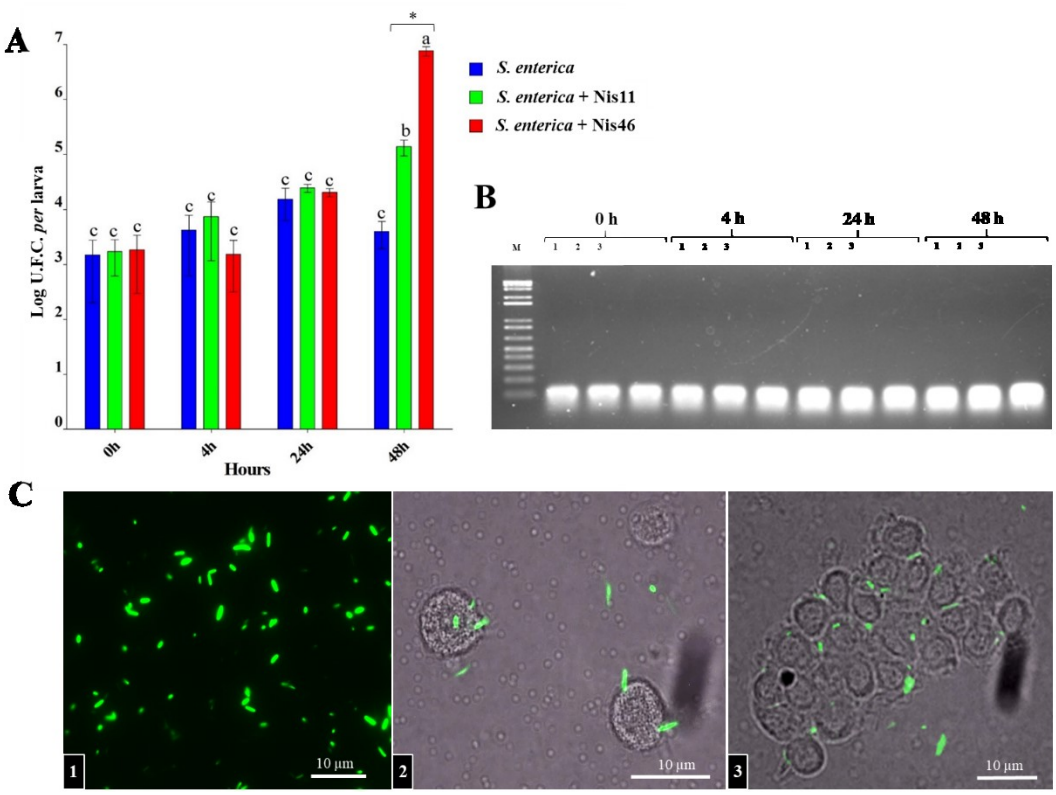

Figure 6: Quantification of *S. enterica* in the hemolymph of *G. mellonella*: 0, 4, 24 and 48 hours post-infection (A). Different letters indicate significant differences by Tukey's HSD test ($p < 0.0001$ PCR of the *invA* gene confirmed the colonies isolated from hemolymph in XLD medium during the same period, where M, Molecular marker 1Kb, 1, *S. enterica*, 2, *S. enterica* + Nis11 and 3, *S. enterica* + Nis46 (B). Exponentially growing *S. enterica*-GFP cells are observed in suspension (C1) and hemolymph of *G. mellonella* after 40 minutes of infection (C2 and C3). The inset in (C2) shows the invasion of the bacterium into the hemocytes. Clumps of hemocytes (nodulation) are observed surrounding the bacterial cells (C3). Bars ≈ 10 µm.

A significant increase in the number of the pathogen after 48 hours of infection in the treatments with *S. enterica* combined with 11.71 µM or 46. 88 µM nisin was registered ($F_{11, 48,} = 130.8$, $p < 0.0001$; Figure 6A). *S. enterica* expressing GFP was seen within infected

larvae, invading, inside or in the periphery of the hemocytes (Figure 6C-2) or in nodules of hemocytes (Figure 6C-3).

The increased of bacterial load on the hemolymph after the infection refers to the ability of this pathogen to overcome the various pathways of larva defense and to be able to invade and multiply within the hemocytes. Pereira et al. (2015) observed a reduces up to four orders of magnitude in CFU of a less virulent strain of *A. pleuropneumoniae* after 4 hours of infection, in contrast, most virulent strain increased by two orders of magnitude 4 hours post-infection, and the cells were still present at 24 hours after larval infection.

Bacteria isolated from hemolymph were confirmed by amplification of the *invA* *Salmonella*-specific gene, and the amplified fragments can be observed on the agarose gel (Figure 6B). As demonstrated in other studies, *invA* is a gene well conserved in all *Salmonella* serotypes, and usually is used for PCR detection (Fey et al., 2004; Oliveira et al., 2013).

The nodules observed in hemolymph during *Salmonella*-GFP infection (Figure 6C) correspond to a natural response of insects to the presence of pathogens, as part of the cellular response (Trevijano-Contador and Zaragoza, 2019). This assay was important to show the ability of *S. enterica* to infect *G. mellonella* hemocytes to cause pathogenicity in the larvae.

The methodology using *S. enterica* expressing GFP was successfully used in previous studies to further elucidate the effect of pathogenic bacteria on the *G. mellonela* model (Bender et al., 2013) and also on the insect *Macrosteles quadrilineatus* (Dundore-Arias et al., 2015), as well as to validate bacterial multiplication in *G. mellonella* by fluorescence measurement by spectrophotometry (Parthuisot et al., 2018). In addition, this method is of great importance for several types of research and can be used, provided that

the expression of the fluorescent markers does not interfere with bacterial virulence (Bender et al., 2003).

3.7 *S. enterica* reduce the number of the hemocytes in *G. mellonella* during infection

The number of the total hemocytes in *G. mellonella* infected with *S. enterica* alone and in combination with nisin exhibit alterations detected from 4 hours after inoculation (Figure 7). This reduction was more evident 24 and 48 hours after inoculation, being potentiated in the larvae infected with *S. enterica* combined with nisin ($F_{5, 24,} = 4.29$, $p = 0.0063$; Figure 7). As expected, the results observed in the control treatments show that the number of hemocytes was maintained during the 48 hours of analysis.

This maintenance of the number of hemocytes is consistent since they are defense cells found in hemolymph and similar to mammalian blood, capable of producing antimicrobial compounds and mediating various defense mechanisms in insects such as coagulation, nodulation, phagocytosis, encapsulation, and melanization (Viegas et al., 2013; Card et al., 2016; Wojda et al., 2017; Scalfaro et al., 2017).

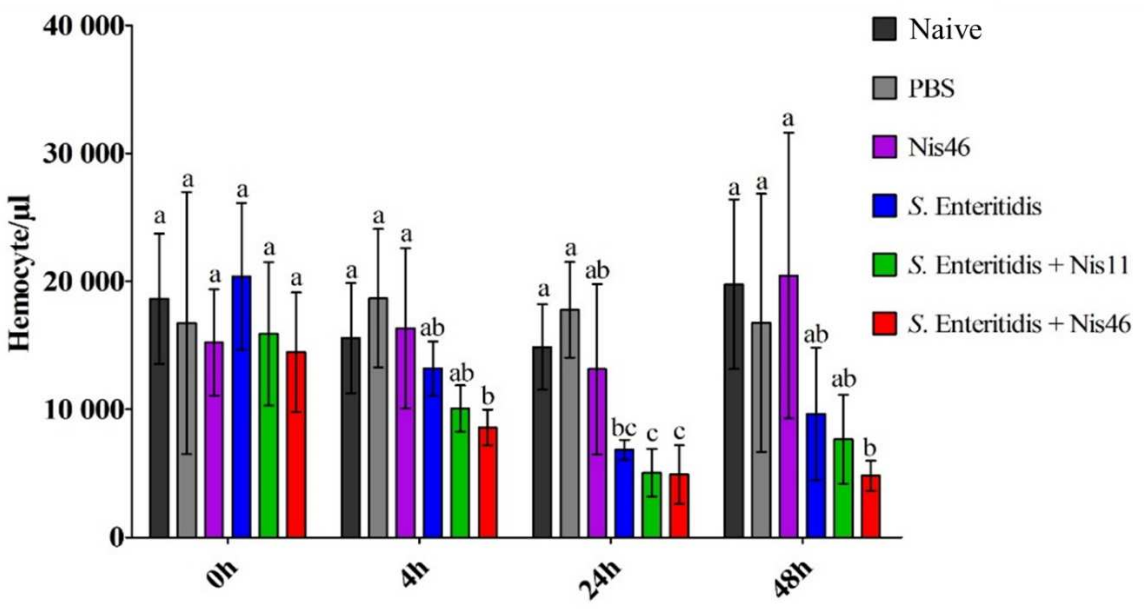

Figure 7: Number of hemocytes in the hemolymph of *G. mellonella* treatments with *S. enterica* at times: 0, 4, 24 and 48 hours post-infection. Different letters indicate significant differences by Tukey's HSD test ($p = 0.0063$).

The reduction in hemocytes number observed in the larvae treated with *S. enterica* combined with nisin may be related to the increased resistance and virulence of these cells described above, and resemble those obtained in studies with *A. pleuropneumoniae* with different degrees of virulence, where the total and differential counts of the immune cells of *G. mellonella* presented different density patterns, with more significant reduction when more virulent strains were inoculated (Pereira et al., 2015). Larvae of *G. mellonella* utilized to evaluate the prophylactic effects of bacteria *Lactobacillus rhamnosus* ATCC 7469 against *Staphylococcus aureus* or *Escherichia coli*, showed a significant increase in the total hemocytes count in all treatments (Jorjão et al., 2018), indicating that the bacteria effectively influence the cellular immune responses of *G. mellonella*.

3.8 *S. enterica* stimulates melanization and emission of pseudopodia in hemocytes of *G. mellonella*

During the dissection of the larvae, it was possible to observe a difference in hemolymph staining between the controls (clear hemolymph) and treated (dark-pigmented hemolymph) larvae which was confirmed by the absorbance analyzes (Figure 8A). Larvae inoculated with *S. enterica* isolated or combined with nisin had more pigmentation (melanin) compared to controls and treatment with nisin ($F_{5, 66} = 36.23$, $p < 0.0001$, Figures 8A and B). The increase of melanin in the hemolymph of infected larvae occurs as a defense response since melanin production is stimulated by the presence of microorganisms (Wojda, 2017; Trevijano-Contador and Zaragoza, 2019). The melanization also occurred in larvae inoculated with *Salmonella* Typhimurium (Bender et al., 2013), *Klebsiella pneumoniae* (Insua et al., 2013), *Legionella pneumophila* (Harding et al., 2012), *S. aureus, E. coli* and *L. rhamnosus* (Jorjão et al., 2018).

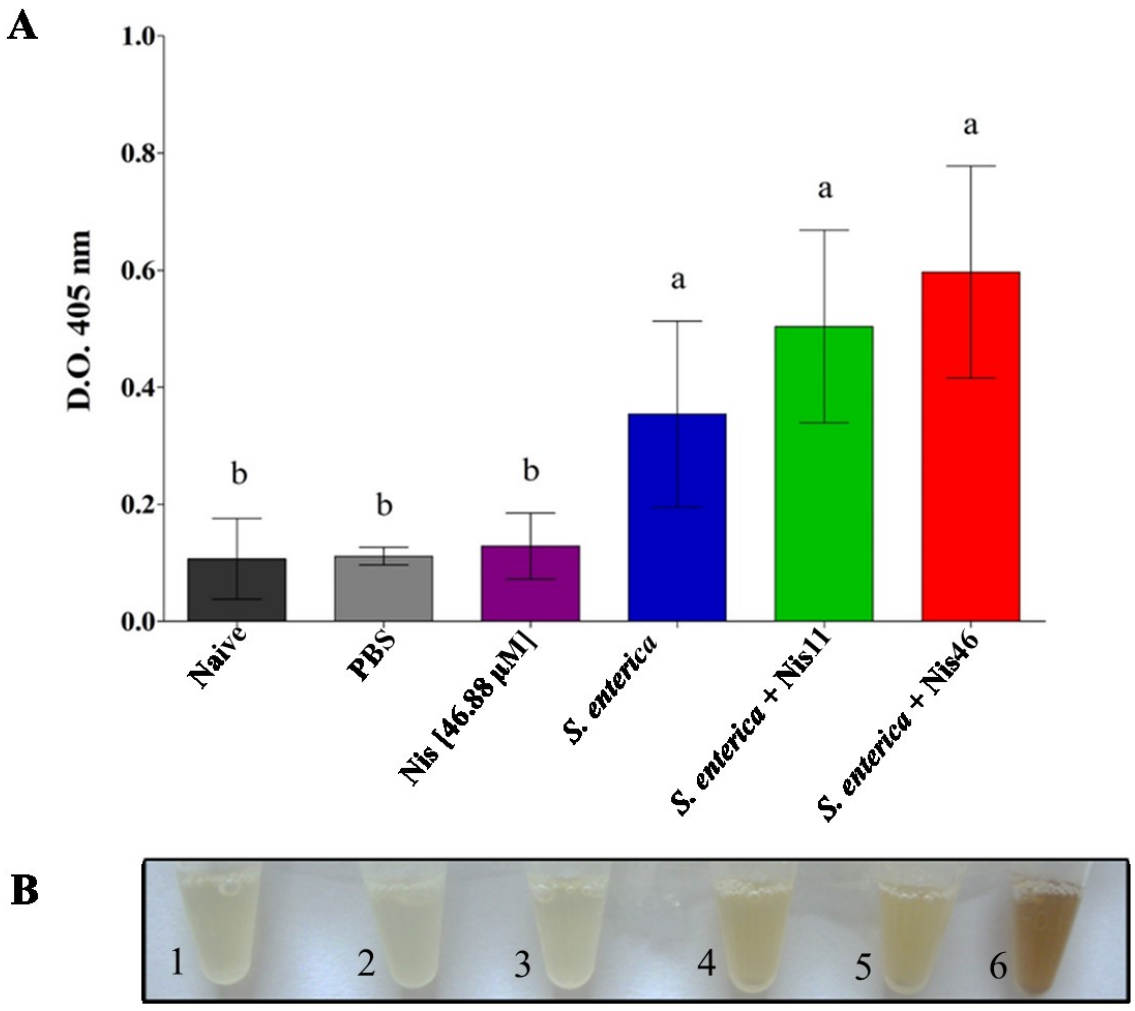

Figure 8: Measurement of black pigmentation of hemolymph of live larvae infected with *S. enterica* 24 hours post-inoculation by the spectrophotometric method (405 nm) in 96-well plates. Different letters indicate significant differences by Tukey's HSD test ($p < 0.0001$) (A). Turbidity of hemolymph diluted in anticoagulant 1: 1, in 1.5 mL microtubes (B), where 1: Not inoculated; 2: Inoculated with PBS pH 7.0; 3: Inoculated with nisin 46.88 µM; 4: Inoculated with *S. enterica* (10^3); 5 e 6: Inoculated with *S. enterica* (10^3) with nisin 11.72 µM and 46.88 µM, respectively.

Morphological analyses showed that in treated larvae, including those inoculated with nisin, occurs the formation of protrusions (or pseudopodia) and melanization nodules in

the hemocytes (Figure 9), essential characteristics for phagocytosis, and encapsulation (Pereira et al., 2015; Blanco et al., 2017). These are a natural process to catch the microorganisms and to reduce infection (Christensen et al., 2005; Tang et al., 2009; Wojda, 2017). Increased nodules of melanization (supplementary material) and pseudopodia were more prominent in the treatments where nisin and *Salmonella* were injected combined to the larvae. In the images of the controls (naive, PBS and nisin) no change was observed in the hemocytes.

In all treatments, including nisin alone, the presence of black pigments (Figure 9) was observed, demonstrating that the immune response was stimulated in an attempt to contain the invaders since these melanin nodules were formed to contain invading microorganisms in the hemolymph (Wojda, 2017; Trevijano-Contador and Zaragoza, 2019).

Fluorescence microscopy confirmed the formation of protrusions previously observed in the histological analyses (Figure 10), and these changes in hemocytes could be induced by the *S. enterica* infection as an attempt to capture the pathogen. Interestingly, cells from treated larvae with *S. enterica* combined with nisin showed more prominent pseudopods (round cells) compared to controls (fusiform cells) (Figure 10). It suggesting that these treatments were more invasive and intensified the defense response of the larva, and that this response is performed by plasmatocytes, a type of hemocyte that exhibits irregular processes, philopodia and pseudopodia in its plasma membrane and have adhesive properties that contribute to the formation of agglomerates, being reported with the function of phagocytosis of invaders (Wu et al., 2016).

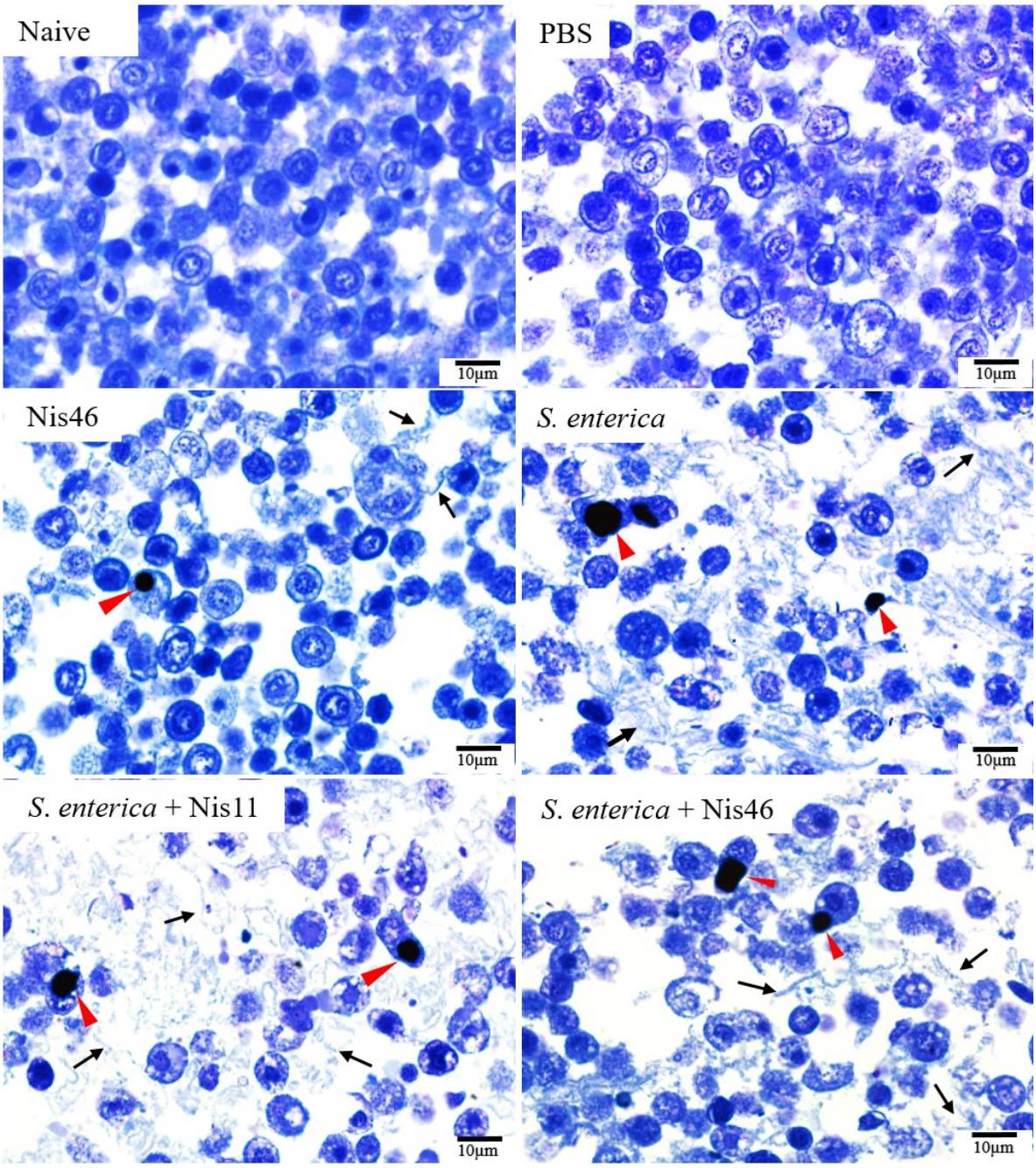

Figure 9: Histological sections of centrifuged hemocytes in *G. mellonella* treated (Nis46, *S. enterica*, *S. enterica* + Nisin11, and *S. enterica* + Nis46) and control (Naïve, and PBS), stained with toluidine blue. Melanization (red arrowheads) and pseudopodia (black arrows) were evidenced in all treated larvae. Bars = 10 µm.

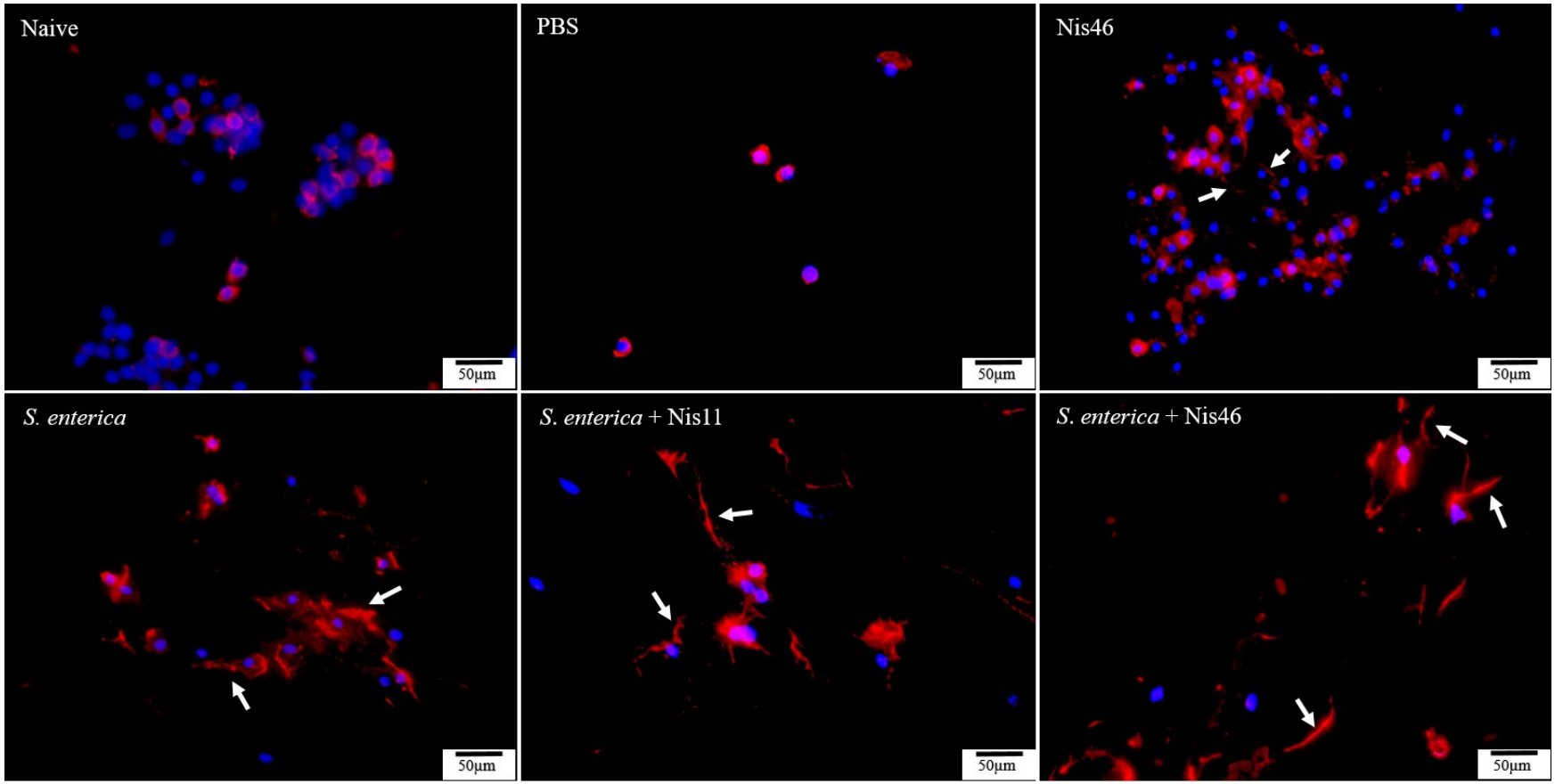

Figure 10: Hemocytes at *G. mellonella* treated (Nis46, *S. enterica*, *S. enterica* + Nisin11 and *S. enterica* + Nis46) and control (Naive and PBS), stained by with 4-6-diamidino-2 phenylindole (DAPI) for nuclei (blue) and Rhodamine Phalloidin for F-actin (red), evidencing the formation of pseudopodia (white arrows). Bars = 50 µm.

The morphological changes in the hemocytes are a response to the presence of pathogenic microorganisms, which can be observed through hemolymph analysis or histopathological examinations. Card et al. (2016) observed, during the infection of 13 serovars different of *S. enterica*, severe damage to the intestinal tissue of the larvae. Transmission electron microscopy also showed the replication of the bacteria in the larval hemolymph, in addition to revealing the presence of vacuoles containing bacteria in the hemocytes, similar to those found in vacuoles visualized in mammalian macrophages (Card et al., 2016).

3.9 Nisin and *S. enterica* reduces the number of hemocytes positive for peroxidases and cactus in *G. mellonella.*

The number of cells positive for peroxidase was lower in treated larvae, both with nisin and, or *Salmonella*, than in the control ($p < 0.001$) (Figure 11A and B). Peroxidases correspond to a large group of oxidizing enzymes, as well as, superoxide dismutase, catalase, glutathione transferase, glutathione reductase, and esterases, which have been characterized in insects. These enzymes play important role in cell detoxification, and their expression change in response to invaders, insecticides, and oxidative stress (Ahmad and Pardini, 1990; Felton and Summers, 1995; Chaurasia et al., 2016; Pereira et al., 2018). The activity of various antioxidant enzymes, such as esterases, multifunctional oxidases, and catalase were significantly affected in the hemolymph of *Locust migratory* treated with *Metarhizium anisopliae* (Cao et al., 2016).

The enzyme cactus presents a similar response to those observed for peroxidase and, treatments with nisin and, or *Salmonella,* reduced the number of cactus-positive cells ($p < 0.001$, Figure 11C and D). *S. enterica* combined with nisin presented a more significant reduction in cells positive for cactus activity (Figure 11C and D).

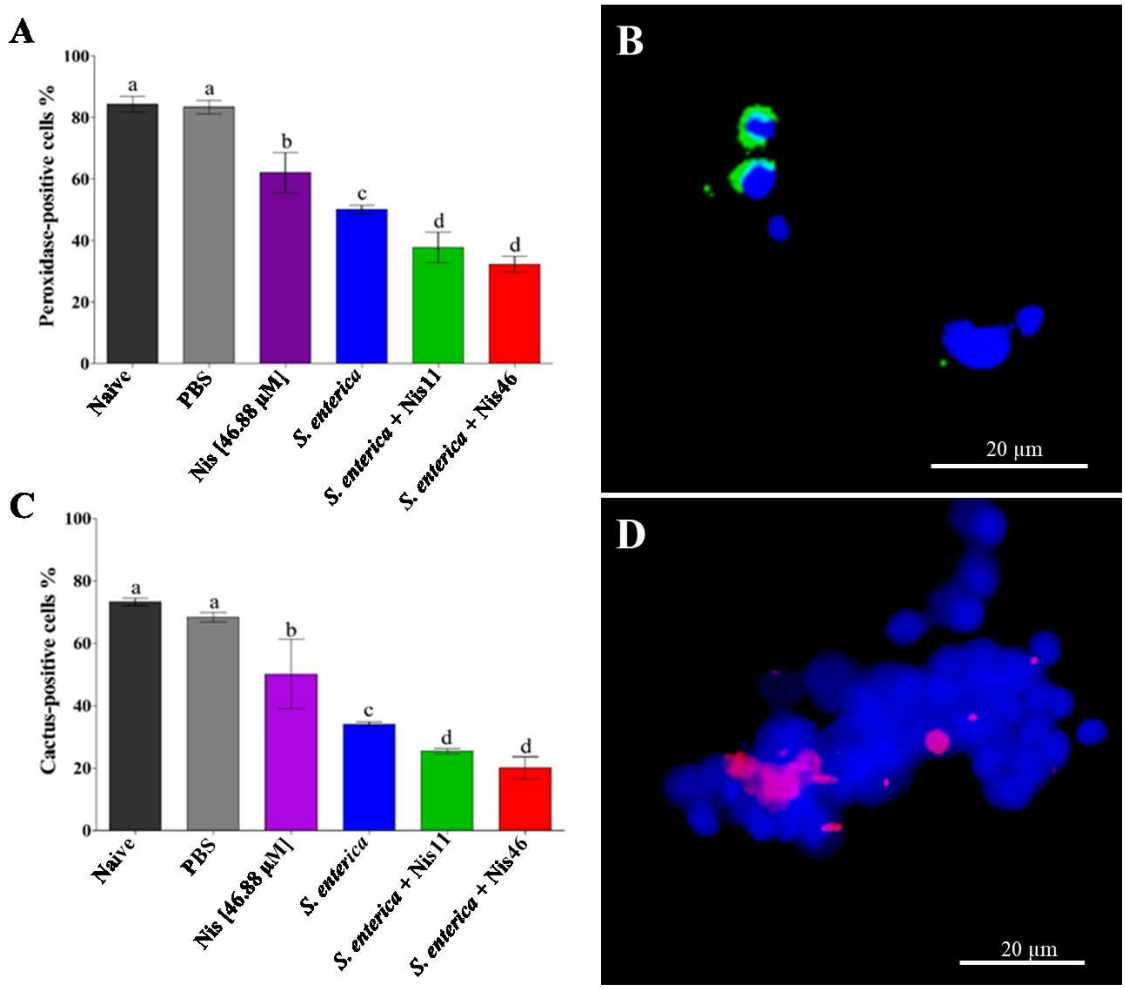

Figure 11: Flow cytometric analyses and immunostaining for peroxidase and cactus in circulating hemocytes of *G. mellonella* treated (Nis46, *S. enterica*, *S. enterica* + Nisin11 and *S. enterica* + Nis46) and control (naïve, and PBS). Percentage of peroxidase-positive and cactus-positive cells; Different letters indicate significant differences by Tukey's HSD test ($p < 0.0001$) (A and C). Cells stained for peroxidase (FITC-green) and cactus (TRITC-red) in hemocytes exposed to *S. enterica*; nuclei are stained with 4-6-diamidino-2 phenylindole (DAPI) (B and D). Bars = 20 µm.

The reduction of cactus-positive cells may be related to the function of this protein, acting as an inhibitor of the transcription factor NF-kB, which is responsible for the transcription of several genes related to the immune system and the synthesis of antimicrobial peptides (AMPs) of the Toll pathway, stimulated in the presence of invading microorganisms (Wu and Anderson, 1998; Hetru and Hoffmann, 2009).

Although the Toll pathway is described for Gram-positive bacterial and fungal infections, in the present study, we demonstrate its participation during *S. enterica* infection in *G. mellonella* hemocytes. Other authors have been observed activation of the Toll pathway in *Tenebrio molitor* larvae treated with *Escherichia coli* (Valanne et al., 2011; Jo et al., 2017).

4 CONCLUSION

Sub-MIC concentrations of nisin are capable of inducing resistance and virulence response in *S. enterica* with increased gene expression usually activated during stress exposure and the invasion in host cells. In addition, the bioassays with *G. mellonella* showed that the pathogen combined with nisin was more effective in the infections, resulting in more significant changes in the immune responses, such as increased melanization, change in cell shape and change in cells expressing protein related to the assembly of the immune response and stress. Thus, it is evidenced that nisin is capable of potentiating the phenotype of virulence in *S. enterica* cells.

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7 CONCLUSÃO GERAL

Em condições sub-inibitória, nisina atuou como molécula sinalizadora de diversos respostas fenotípicas de *S. enterica*, tais como no aumento da virulência, formação do biofilme e expressão gênica de transcritos envolvidos com a virulência, resistência e biofilme, normalmente expressos durante a exposição à agentes estressores e o processo de invasão das células hospedeiras. As análises combinatórias entre nisina sub-inibitória e os agentes estressores baixo pH, elevadas concentrações de NaCl e antibióticos aumentaram a atividade inibitória da nisina sobre *S. enterica*, demostrando o potencial biológico desse peptídeo em bactérias Gram-negativas.

S. enterica associada à nisina em concentrações sub-inibitória estimulou alterações nas respostas imunes durante os bioensaios com *G. mellonella,* resultando em aumento da mortalidade, melanização, protrusões (pseudópodes) e proteínas relacionadas à montagem do sistema imunológico, especialmente na maior concentração utilizada.

Os resultados apresentados são inéditos, e demonstram que a concentração sub-inibitória de nisina é capaz de alterar o comportamento de *S. enterica* potencializando sua virulência que são possíveis de serem demostradas em larvas de *Galleria mellonella*. Esses dados aumentam o entendimento da ação da nisina como molécula sinalizadora de importantes funções dentro de um contexto ecológico microbiano.